ACTA PHYSIOLOGICA SCANDINAVICA
VOL. 29. SUPPLEMENTUM 107

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ON THE PLACENTAL TRANSFER OF IRON

AN EXPERIMENTAL STUDY IN THE RAT

BY

GUNNAR NYLANDER

UPPSALA 1953

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FROM THE DEPARTMENT OF HISTOLOGY (HEAD: MARTIN WRETE, M. D., PROFESSOR OF HISTOLOGY, UNIVERSITY OF UPPSALA)

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AKADEMISK AVHANDLING

som med tillstånd av
Medicinska Fakulteten vid Uppsala Universitet
för ernående av medicine doktorsgrad offentligen försvaras
å Histologiska institutionen tisdagen den 26 maj 1953
kl. 10 f. m.

UPPSALA 1953

ALMQVIST & WIKSELLS BOKTRYCKERI AB

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Introduction and acknowledgements

How iron is transmitted from the maternal to the foetal circulation is a problem that has attracted less attention in the last decades than other branches of iron metabolism of the body. Earlier the problem was dealt with mainly by histological and histochemical technique, but, with the detection of the serum iron chemical determinations of maternal and foetal serum iron concentrations have revealed new facts from which conclusions about the iron transfer mechanism can be drawn. Other advances in this field have been made possible by the introduction of radioactive tracer substances into Medical Science, and the detection of the iron-binding protein of the serum. The concentrations of serum iron and the iron-binding protein of the serum in maternal and foetal blood have thus been recorded, and they indicate that a transfer process across the placenta exists which differs from diffusion. Similar conclusions have been drawn by means of tracer studies using radioactive isotopes of iron. Further, the maternal serum iron has definitely been shown to be the source of foetal iron.

The placental transfer of iron from the maternal to the foetal circulation is made up by a process the starting-point of which is the maternal, and the end-point the foetal, serum iron complex. The nature of this process is, however, still unknown.

The present investigation, an experimental study in the rat, is intended as a contribution to our knowledge about this transfer process. Normal and iron-deficient test animals in different stages of pregnancy have been used. Firstly, histochemical investigations have been carried out on placental structures with special reference to iron, secondly, the placental and foetal contents of an experimentally chosen iron fraction have been determined, finally, some experiments on placental transfer by means of radioactive iron are included.

The experimental work was carried out at the Histological Department, University of Uppsala. I wish to thank the head, Professor Martin Wrete, M.D., for the privilege of working at his Department, and for his co-operation and interest.

My thanks are due also to Professor JOHN NAESLUND, M.D., my former chief, whose inspiring interest in research stimulated me to begin this investigation.

It is a pleasure here to thank Associate Professor CARL-BERTIL LAURELL, M.D., for his valuable suggestions and advice.

Associate Professor SVEN BROLIN, M.D., has throughout followed my work with unfailing interest.

In the statistical analysis Mr ELVIR LANDER, Actuary, has given me invaluable help, for which I take the opportunity of thanking him.

It is a pleasant duty to thank my technical assistants, Mrs Aino Ilmjärv and my wife Karin for their exceptional energy and skill.

I am especially grateful to the staff of the Department of Histology for various kinds of technical assistance.

Mr REGINALD STEDMAN revised the English manuscript and for this I owe him my sincere thanks.

I have received financial assistance from Statens Medicinska Forskningsråd, from the Medical Faculty of the University of Uppsala, and from Magnus Bergwalls stiftelse, and I wish to render to the various institutes my thanks.

Uppsala March 1953 *Gunnar Nylander*

CHAPTER I

Features of the iron metabolism

The iron metabolism in mammals is the subject of a comprehensive literature, much of which is interesting from the historical point of view, although, the essential features may be derived from the results obtained from the research of the last twenty years or so. Much of this new knowledge has been obtained through the use of artificial radioactive isotopes of iron, making it possible to establish the regulation of iron absorption and excretion, the metabolism of haemoglobin and other chromoproteins, the transport of iron within the body and the physiology of the iron depots. Other milestones in this field of research are the detection of the storage-iron fraction, ferritin, and the iron-binding protein of the serum.

It is not my intention to give a survey of the abundant literature; instead, I will content myself with mentioning some reviews of it and will refer directly only to articles contributing information essential to the exposition of current views on the iron metabolism.

A. Different iron compounds and their distribution in the organism

Iron is a vital constituent of every mammalian body cell. The different iron compounds in mammalian tissues may be separated chemically as well as biologically into three fractions: *the chromoprotein iron*, *the storage-iron*, and *the serum iron*. The proportions of these fractions are given by GRANICK, 1946, for a 70 kg man with an average of 4.3 gm of iron. Some 65 % is contained in the chromoproteins and the remaining 30—35 % of the body iron is designated the storage-iron fraction. The serum iron fraction is calculated to be about 1 ‰ of the total body iron. DRABKIN, 1951, concludes that about 76 % of the total iron in a 70 kg man is in the chromoproteins and about 16.5 % in the two iron protein complexes of storage and serum iron.

1. Chromoproteins

This fraction represents the highest potency of biological activity. Chemically, the iron compounds of this fraction are defined as haeme or haemine proteins, the iron occurring as the ferrous or ferric salt of the prosthetic porphyrine group. Most of these iron compounds are included in the haemoglobin of the red blood cells, and to a lesser degree in the myoglobin of striated muscles. Another group of chromoproteins of vital importance is the cell haemins or the biocatalysts cytochromes, catalases and peroxidases involved in the oxidation of cell metabolites in every mammalian cell. The haemoglobin turnover is of paramount importance in the intermediary iron metabolism. Physiologically, there is a continous breakdown and resynthesis of red blood corpuscles in the body. On the basis of figures recently given for red cell and serum iron turnover, Laurell, 1952, suggests that some 75 % of the total serum iron turnover is derived from the destruction of red blood cells. This makes it clear that the utilization of the iron contained in haemoglobin is much more effective in blood regeneration than that of absorbed iron or storage-iron.

2. Storage-iron fraction

This iron fraction may be considered as the body's reserve of iron, available when need arises for the element, and thus temporarily out of 'biological action,' — inert iron. Studies in man would indicate normal iron reserves of 1 200—1 500 mg in the adult male (Haskins, Stevens Jr, Finch, and Finch, 1952). Our knowledge of the chemistry and physiology of the storage-iron fraction has been greatly extended during the last decade. The main part of this fraction is now considered to be *ferritin*, an iron-protein complex isolated in 1937 by Laufberger from the spleen and liver of the horse. Since then, there have been many contributions to the rather extensive literature on this subject. The reviews of Granick, 1946 and 1951, and Michaelis, 1947 may be consulted for further references.

Ferritin is an iron-protein complex, the protein component of which is called apoferritin. This homogeneous protein has a large molecular weight (465 000) and is crystallisable with cadmium sulphate in the form of colourless crystals. The iron is in the form of colloidal ferric hydroxide-phosphate complexes, which are taken to be bound in a rather firm way to the surface of the protein

by an obscure chemical linkage as micelles. The variable size of the iron micelles would account for the varying iron content of ferritin (17—23 % by dry weight). Iron is in the trivalent state, but has 3 unpaired electrons, and in this respect it is different from all other forms of body iron, from which it can thus be readily distinguished magnetometrically. Iron as it occurs in ferritin is ordinarily insoluble, but the complex it forms with the protein to which it is attached has a stable, soluble molecule. Ferritin may be crystallised in the same way as apoferritin with cadmium sulphate, giving yellow-brown polyhedric crystals.

The storage function of ferritin is suggested by its high iron content and the fact that the iron is in an innocuous form within a soluble molecule. Definite proof of this function of ferritin has been given by Hahn, Granick, Bale, and Michaelis, 1943, and Granick and Hahn, 1944, who found isotopically labelled ferric salt to be incorporated in the ferritin fraction of the liver. The injected iron had 5 unpaired electrons, but the iron contained in the ferritin fraction had been converted into the 3 unpaired electron type of the ferritin iron. Granick, 1946, after oral administration of iron to guinea pigs, found an increased amount of ferritin in the intestinal mucosa and in the liver. As there was no ferritin, or very little, present before the administration of iron, it was concluded that the feeding of iron results in an increase of the specific protein apoferritin. It is further pointed out by Granick, 1951, that "the synthesis of ferritin in response to iron is a phenomenon not limited to the cells of the gastrointestinal mucosa. It seems to be a property of a number of tissues . . . In general, those tissues which contain iron-staining granules and therefore have a high content of iron will be found to contain a high content of ferritin, the amounts of iron running parallel to the amount of ferritin."

When there is great demand for iron in the haemoglobin synthesis, on the other hand, the storage-iron is mobilised. The greatest depot of iron, the liver, gives up ferritin and apoferritin, as shown by Granick, 1943, in horses bled for serum. Granick therefore suggests that ferritin might be regarded not only as a storehouse of body iron but of protein as well (Granick, 1951). Agner, 1943, reported that red blood cells of the horse contain ferritin. Normally, however, ferritin has not been found to be present in the serum, and consequently does not leave the depot organ as such. It has to be split up in order to make iron available (possibly in an ionised form?) to the iron-binding protein of the serum. How this is accomplished is not known. Braunsteiner, Gisinger, and Pakesh, 1952, advance a theory that there

exists some sort of a dynamic equilibrium between the proteins of apoferritin in the depots and the iron-binding protein in the serum. This, has not been confirmed, however, and LAURELL, 1947, states that the iron is bound differently in the ferritin and in the serum iron-protein complex.

It thus seems to be an established fact that ferritin serves to store iron which may be mobilised when there is increased demand for iron, and accumulated in the reverse circumstances. The amount of ferritin might in this way reflect the iron balance of the body. It has been pointed out by ROTH, JASIŃSKI, and V. BIDDER, 1951, and HASKINS, STEVENS JR, FINCH, and FINCH, 1952, that depleted iron stores are to be regarded as a 'preclinical phase' of iron deficiency.

In the past, *haemosidern* was considered to be the main storage-iron compound in the body. Today it is no longer assigned such a prominent place in this respect, but it is not to be neglected. It is known to exist in the same tissues as ferritin. It seems to represent iron in excess, rather than a physiological iron-storage fraction. Its chemical constitution is not known, but there are two theories as to its structure. COOK, 1929, thinks it is colloidal ferric oxide or hydroxide attached to the morphological stroma. GRANICK, 1949, suggests that it is a polymerisation product of ferritin. DRABKIN, 1951, says that "haemosiderin most probably represents tissue iron in excess of the capacity of apoferritin to 'bind' it." Haemosiderin is histochemically demonstrable in the tissues. Unstained it is seen as a yellow granular precipitate, and with the different iron reagents of histochemistry it appears as coloured granules.

Metabolic pool of iron. GREENBERG and WINTROBE, 1946, suggest the existence of a 'labile pool of iron' which might be separated from the ordinary iron stores. DRABKIN, 1951, discussing this theory, considers that only a fraction of the storage iron is available to the active metabolic pool, and points out that this labile store of iron might be in an other form than ferritin. BRAUNSTEINER, GISINGER, and PAKESCH, 1952, point out that there seems to be a continuous exchange of iron with some fraction postulated to exist in the reticulo-endothelial system and controlled by the adrenal cortex, definitely separated from the storage iron fraction, and considered as a 'metabolic pool of iron'.

3. Serum iron

Serum or plasma iron is the iron of transport between the different tissues of the body. After the absorption of iron in the gastrointestinal mucosa, the element enters the blood stream and from now on its

further migration within the body — to the sites of haemoglobin, myoglobin and enzymatic iron synthesis, and to the sites of storage — occurs in the form of serum iron. The same applies to the iron liberated in the catabolism of different chromoproteins — above all haemoglobin — and to the iron mobilised from the depots.

The honour of having detected this form of iron is ascribed to Fontés and Thivolle, 1925, who were the first to demonstrate the presence of iron in horse serum. Other articles of historical importance are those of Barkan, 1927, Warburg, 1927, and Warburg and Krebs, 1927. The literature concerning serum iron is today overwhelming. The reader is referred to the numerous monographs. (Heilmeyer and Plötner, 1937; Moore and coll. 1937–39; Skouge, 1939; Albers, 1941; Vahlquist, 1941; Vanotti and Delachaux, 1942, 1949; Laurell, 1947, 1952; Lundström, 1949; Rustung, 1949; Tötterman, 1949; Andersson, 1950; Hemmeler, 1951.)

The serum iron is known as an iron-protein complex of high molecular weight, which is stable at the pH of the blood. Under these conditions, the complex is non-dialysable and not dissociated, or only to an insignificant extent. As the acidity increases beyond neutrality the complex becomes dissociated to an increasing degree, and ionised iron is found in the ultrafiltrate in dialysis experiments. Serum iron is thus hydrolysable, acid-soluble iron.

The iron-protein complex constituting the serum iron contains iron in the trivalent state. This was suggested long ago by Starkenstein and Weden, 1928, and others, but was only recently verified by magnetic susceptibility measurements (Surgenor, Koechlin, and Strong, 1949). The latter authors also state that the bond between iron and protein is of an ionic nature. The specific iron-binding protein — transferrin according to Laurell's nomenclature — has been thoroughly studied with regard to both its physical and biochemical identities. Laurell, who has contributed much of our knowledge in this field, gives a list of relevant literature in an extensive review (Laurell, 1952). It has been shown to have a molecular weight of about 90 000 (Laurell and Ingelman, 1947, and Surgenor and coll., 1949), thus only a little greater than the albumins. The protein has been isolated from Cohn's fraction IV: 7 when purified and concentrated (Surgenor, Strong, Taylor, Gordon, and Gibson, 1949); electrophoretically the protein moves with the β_1-globulins and is accordingly called "metal-combining globulin." Laurell points out, however, that in salting out experiments the protein is soluble in half-saturated ammonium sulphate solution, like the albumins. It has also been suggested by Braunsteiner, Gisinger, and Pakesch, 1952, that the small molecular transferrin seems to follow the albumins rather than the other large molecular β-globulins in its quantitative variations under pathological conditions. Transferrin in the iron-

free state has a great affinity for iron, — greater than for copper and zinc — and each molecule of transferrin is said to be able to bind two atoms of iron (Laurell and Ingelman, 1947). The groups responsible for this affinity for iron have not been identified. Of great importance is the statement of Laurell, 1947, that a partial dissociation of the iron-transferrin complex occurs at a pH as high as 7 in the presence of serum. This means that the complex must be to some extent dissociated under physiological conditions in the blood.

Holmberg and Laurell, 1945, and more comprehensively Laurell, 1947, were able to show that beside the protein fraction in the serum iron which corresponds to the "manifest iron-binding capacity" of the serum, there is an additional protein fraction capable of identical binding of iron added in vitro and in vivo. This protein fraction represents "the latent iron-binding capacity" of the serum. The sum of these two fractions provides us with an estimate of "the total iron-binding capacity" of the serum, which is alternatively termed "the saturation limit of the serum." Iron added in excess of the capacity of available transferrin to bind it, i.e. above the saturation limit of the serum, will be only 'loosely bound,' and dialysable. The total iron-binding capacity of 100 normal subjects of both sexes averaged 315 γ % in the material of Laurell, 1947. As the normal serum iron level is about 100 γ %, it follows that only about one-third of the total iron-binding capacity of the serum is utilised, so that transferrin exists in the serum in considerable excess compared to the normal need for an iron vehicle. As mentioned earlier, Laurell, 1947 and 1952, stresses that there is some dissociation of the iron-transferrin complex even within physiological pH ranges of the blood. Furthermore, Laurell has shown that the quotient, manifest iron-binding capacity of the serum / latent ditto, is intimately correlated to changes in the intermediary iron metabolism. This leads him to suggest that "it seems rather reasonable to assume that the iron-binding component of the serum serves as a balancing regulator for the exchange of iron between various organs of the body." He further suggests that a possible explanation of this regulating rôle of the iron-binding component of serum in the intermediary iron metabolism might be found in the equilibrium maintained in the serum between the activity of iron ions, iron-free transferrin and the iron-transferrin complex. He expresses this equilibrium by the equation:

$$\frac{\text{Fe-transferrin}}{\text{Fe-free transferrin}} = K\,(Fe^{+++}).$$

Commenting on this equation, Laurell states: "The influence of the transferrin level on the concentration of ionised iron in plasma can be comprehended from this formula, and further it is clear that the plasma in this way mediates an equilibrium between the iron ion activity in the different organs of the body. This hypothesis explains why iron is mobilised from the iron stores during increased demand in the bone marrow and during pregnancy when the iron is lost to the fetus. Under these conditions, the iron ion activity decreases in plasma and a concentration fall is established between the body cells and the plasma."

B. The iron metabolism in non-pregnant conditions

1. Excretion of iron

The ability of the organism to excrete iron is extremely poor. Balance studies have confirmed this opinion (Lintzel, 1927, 1929; Farrar and Goldhamer, 1935; Widdowson and McCance, 1937, 1938, 1943 and others), and so do the results of experiments with radioactively tagged iron (Hahn, Bale, Hettig, Kamen, and Whipple, 1939; Greenberg, Copp, and Cuthbertson, 1943; Copp and Greenberg, 1946; and Dubach, Moore, and Minnich, 1946). The view that considerable amounts of iron are eliminated with the sweat (Mitchell and Hamilton, 1949) has not been confirmed (Stewart, Snowman, Yuile, and Whipple, 1950; and Adams, Leslie, and Levin, 1950). The overwhelming part of iron once incorporated in the body will therefore remain in the endogenous iron metabolism. It has earlier been pointed out that the continuous breakdown and resynthesis of red blood cell haemoglobin, the cycle haemoglobin — serum iron — haemoglobin, comprises about 75 % of the serum iron turnover, the remaining part being contributed by the catabolism of other chromoproteins than haemoglobin, the absorbed iron and the storage-iron fraction.

2. Absorption of iron

So, normally, the absorption of iron from the intestine must remain very small, and the mobilisation of the iron depots must be extremely insignificant. But when the demand arises for an accelerated erythrogenesis or iron deposition in the conception product, an increased

absorption and mobilisation of iron from the depots commences. The iron absorption is regulated to avoid both iron excess and iron deficiency.

An American research team associated with HAHN has definitely established the poor absorption of iron from the gut in normal conditions, the intestinal mucosa keeping the element out. In anaemic or, strictly speaking, iron-deficient conditions brought about by bleeding or by iron-deficient diets, the absorption is considerably increased in the dog (HAHN, BALE, LAWRENCE, and WHIPPLE, 1939) in the rat (COPP and GREENBERG, 1946), and in man (HAHN, 1948). This could not be attributed to the anaemia per se, as the increased absorption did not occur in untreated pernicious anaemia (BALFOUR, HAHN, BALE, POMMERENKE, and WHIPPLE, 1942). Moreover, in the acute anaemia produced by bleeding, it did not appear until the anaemia was overcome, but then amounted to 5 —10 times the normal absorption (HAHN, BALE, ROSS, BALFOUR, and WHIPPLE, 1943). Some hours after the oral administration of an iron dose, they were able to register a cessation of further absorption, and they introduced the term '*mucosal bloc*' to describe this. The duration of this bloc in normal dogs was several days, but in anaemic animals of short duration, 18—20 hours (STEWART, YUILE, CLAIBORNE, SNOWMAN, and WHIPPLE, 1950). An explanation of the probable nature of this regulative mechanism has been put forward by GRANICK, 1946. He was able to show that ferritin is found in the duodenal mucosa of the guinea pig in normal conditions, and that the amount of ferritin is considerably increased after iron feeding. This increase reaches a maximum after 7 hours, and then gradually decreases to normal values within 3—6 days. On the basis of these findings, coupled with the results of absorption studies referred to above, he ascribes the regulating mechanism of the gastrointestinal mucosa to ferritin. In the reviews of 1949 and 1951, Granick deals with the supposed regulating function of ferritin in intestinal absorption of iron, and in the latest one he states: "Whether changes in ferritin concentration are directly connected with the mucosal bloc, or whether the presence of much ferritin merely indicates that the mucosal cell is saturated with respect to some iron compounds more closely allied with the mucosal bloc, has not been determined."

Thus it must be taken as established that under normal conditions the absorption of iron from the intestine is definitely limited by a

regulative mechanism of the intestinal mucosa. The nature of this mechanism may possibly be that outlined by Granick, but it must still be considered obscure.

To sum up, the main features of the iron metabolism under non-pregnant conditions are endogenous, within the cycle, breakdown of haemoglobin — serum iron — resynthesis of haemoglobin. The additional amounts of iron needed are insignificant, the absorption from the intestine regulated and small, and mobilisation from the iron depots little or none. In conditions of increased iron demand, on the other hand, the absorption from the intestine and the mobilisation of iron available in the depots are considerable. The adjustments in iron metabolism caused by an iron-deficiency — increased iron absorption, increased iron mobilisation from the depots — might be brought about in accordance with Laurell's theory already referred to.

3. Sexual differences in the iron metabolism

Certain sexual differences in the iron metabolism might be of interest regarding the rôle of the female in reproduction. It has been known for some time that women have lower haemoglobin values than men, apart from the calculated iron loss with menstrual bleeding. This was definitely established by Vahlquist, 1944, who found the same low value of female haemoglobin in women of fertile age who had undergone uterine amputation.

The same intersexual difference exists in the serum iron concentration. From investigations of the last ten years or so the difference seems to be about 10—15 % of the mean male serum iron value. (Skouge, 1939; Vahlquist, 1941; Hemmeler, 1946; Laurell, 1947; and Tötterman, 1949).

The question of whether or not this difference is a consequence of the iron lost by menses in the women has, like the similar question regarding haemoglobin concentration, been answered by Vahlquist, 1944, who observed in the women previously mentioned the same difference as in normal women. The cause of this difference is still obscure, however (Vahlquist, 1950; Hemmeler; 1951). The transferrin concentration of the serum does not show the same sort of difference between the two sexes (Laurell, 1947; Rath and Finch, 1949; and Hagberg, 1953). Cartwright, Black, and Wintrobe, 1949, found women to have somewhat higher values than men.

In the female rat, there seems to be a higher deposition of iron in the liver than in the male animal. This was shown by Steenbock, Semb, and Van Donk, 1936. They also noted that castration eliminated this difference. In young animals, not sexually mature, the same deposition of iron in the liver could be induced by injections of follicular hormone. The iron depots of the female liver were exhausted during pregnancy. These results were later confirmed by Widdowson and McCance, 1948. The depot fraction of the liver was of the same magnitude in both sexes up to commencement of sexual development. From that time on, the female rat continued to store more iron in the liver than the male — up to 3 times as much. These results could not be reproduced in the rabbit or the guinea pig. It thus seems that there is in the rat a presumably hormonally regulated deposition of iron in the female's liver as a preparation for pregnancy, but this is not the case in other species investigated.

In man, the contrary seems to be the case (Lapique, 1947; Schairer and Rechenberger, 1948). Roth, Jasiński, and v. Bidder, 1951, found the storage-iron fraction per unit weight of liver, spleen and muscles of human section material to be considerably lower in women than in men, and ascribe this difference to the iron loss with menstrual bleeding in women.

C. The iron metabolism during pregnancy

1. Iron requirements during pregnancy

The demands on the maternal metabolism in pregnancy are considerable. Iron is continuously required for the growth of the uterus and the development of the conceptus. The iron content of a mature human foetus has been determined as about 274 mg (Widdowson and Spray, 1951, the values varying from 201 to 372 mg). Earlier investigators have presented results of the same order of magnitude (Hugounenq, 1899; Jerlow, 1934; Swanson and Iob, 1939; and others). It is pointed out by Iob and Swanson, 1938, that the percentage of foetal iron per weight unit of fat-free and water-free substance is practically unchanged during the period of foetal growth. The amounts of iron per unit weight of fat-free foetal body substance, as given by Widdowson and Spray, seem to indicate an increase with

development, however. But in either case the main part of the foetal iron is acquired during the last three months of pregnancy.

The total maternal loss of iron caused by pregnancy and parturition depends largely on the amount of blood lost as intrapartum haemorrhage. Consequently, the values given in the literature differ considerably, especially as the lactation period is sometimes taken into account. The figures given vary from 600 mg (Heath and Patek, 1937) to 900 mg (Coons, 1932; Fullerton, 1936).

Rath, Caton, Reid, Finch, and Conroy, 1950, on the basis of figures given in the literature, estimate the iron lost to the foetus during pregnancy as 400 mg, but referring to an increase in the total circulating red cell mass occurring in pregnancy — suggested by Gibson and Caton, 1950 and corresponding to some 500 mg of iron — they found the maternal debit of iron to be 900 mg.

The amount of iron lost during normal mestruation has been found to be approximately 20 mg (Barer and Fowler, 1936), which would require an average daily intake of 0.7 mg during a 28 days cycle. On the basis of these figures, Vahlquist, 1944, states that the amount of iron delivered to the conceptus, expressed as mg of iron per day is smaller during the first half of pregnancy than the physiological loss of iron in the menses expressed in the same way. Thus, far from losing iron on account of the uterine content, the mother has at this stage of pregnancy a more positive iron balance than under normal non-pregnant conditions. During the latter half of pregnancy, on the other hand, the daily iron requirements of the conceptus far exceed the gain in iron corresponding to the absence of menstrual bleedings. The demand for iron during this stage of pregnancy is considerable, and to meet this increased need for iron the maternal organism has to adjust its iron intake as well as the intermediary iron metabolism.

2. Iron intake during pregnancy

Hahn and coll., 1947—51, used radioiron to study iron absorption in pregnant women, and found that the intake was much larger during the latter part of gestation. They state in their last article, 1951: "As gestation progressed the uptake of iron increased to the extent that at 30 weeks' gestation and over, three or more times as much iron was absorbed as during the period before the 15th week of gestation."

3. Intermediary iron metabolism during pregnancy

It is now accepted that the serum iron concentration decreases during the latter half of gestation. SUNDELIN, 1942, found the serum iron values to be normal during the first 5 months of pregnancy, after which there was a decrease during the following months, with the lowest value, 48 γ %, in the 8th and 9th months. In the 10th month, however, the serum iron level rose again (80 γ %). RENAER, 1942, found the same tendency, though less pronounced, as did LAURELL, 1947. LUNDSTRÖM, 1949; FAY, CARTWRIGHT, and WINTROBE, 1949; and VENTURA and KLOPPER, 1951, recorded a decrease of the serum iron level in the latter months of pregnancy. This was not noted by RATH and coll., 1950, however.

In conformity with these findings, LAURELL, 1947, determining the capacity of serum to bind iron in different stages of pregnancy, was able to show that this capacity during the first 5 months falls within the limits of normal variation, but markedly increases from the 6th month on, the maximum being attained in the 9th month of pregnancy. This was later confirmed by FAY and coll., 1949, and VENTURA and KLOPPER, 1951. RATH and coll., 1950, did not find this tendency, however, and postulate that geographical circumstances might contribute to an iron deficiency during pregnancy, explaining the different findings.

Thus the serum iron concentration, i.e. the manifest iron-binding capacity of the serum, is within normal limits during the first half of pregnancy, as is the total and consequently the latent iron-binding capacity. In the latter half of pregnancy, however, a decrease of the serum iron level is accompanied by an increase in the total iron-binding capacity of about 200 γ %, with a corresponding increase of the latent iron-binding capacity of the serum. Summing up, and correlating the features of iron metabolism in pregnancy with his findings, Laurell states: "In the last months of pregnancy the latent iron-binding capacity of the serum is about twice as great as normally, and the serum iron is normal or slightly subnormal, i.e. the quotient manifest/latent iron-binding capacity is smaller than normally. The physiological significance of this may very well be that the lower the quotient the more rapidly is iron eliminated from the intestinal canal and the depots."

4. Iron depots during pregnancy

It was suggested by Bunge, 1899, that the high incidence of chlorosis in young girls was explained by the diversion of iron from the circulating blood to the iron depots in order, he considered, to meet the requirements of reproduction. Lately, however, Lapique, 1947; Schairer and Rechenberger, 1948; and Roth and coll., 1951, have found the storage-iron fraction in women to be considerably smaller than in men, as already mentioned. Pregnancy has been proved to deplete the iron stores of female rats (Smythe and Miller, 1929; Steenbock, Semb, and Van Donk, 1936; and Widdowson and McCance, 1948). The latter authors found the percentage of iron in the liver of the female rats to decline rapidly during pregnancy. They further point out that the amount of iron contained in a newborn litter approximately equals the amount of iron lost from the maternal liver, and suggest that most of the iron in the newborn animals may have come from that source. Hahn and coll., 1951, noted differences in iron uptake with parity, multiparous women having a significantly higher mean uptake of iron during the first 19 weeks of gestation than the primiparous. From the 20th week on, however, parity has no influence upon the uptake of iron. They suggest: "It is conceivable that they (the differences in iron uptake) reflect lower iron stores in the multiparous women, and that the normally increased uptake late in pregnancy masks this finding during the latter half of gestation." Rath and coll., 1950, point out that the iron needs of human pregnancy are well met by the normal woman through increased intestinal absorption and the possibility of mobilising some 1 000 mg of depot iron.

Summing up, it is observed that in pregnancy the maternal organism has to meet augmented requirements for iron only in the last half of the gestation period. The adjustments in the mother's metabolism involve increased intestinal absorption, presumably brought about by a considerably elevated latent iron-binding capacity and decreased iron ion activity of the serum. This means that iron entering the circulation will find an increased concentration of iron-free transferrin to bind it. The same mechanism may be responsible for the mobilisation of iron from the depots which obviously occurs in pregnancy.

5. Iron transfer from mother to foetus

The probable sources of foetal iron are 1) the iron absorbed from the intestinal canal of the mother, 2) the iron from her depots and 3) her circulating blood iron.

1) Fetzer, 1913, established in female rabbits a correlation between the female's intake of iron and the iron content of the young. Several authors have likewise noted that the second generation of rats fed an iron deficient diet become anaemic during the first weeks after birth (Happ, 1922; Scott, 1923; Mithell and Schmidt, 1926; Parsons and Hickmans, 1933). Alt, 1938, fed rats an iron deficient diet and found the first litter offspring to show a marked reduction in the total body iron, and the second a decrease of the haemoglobin concentration of the blood as well.

2) As regards the maternal depots of iron, it has earlier been pointed out that their depletion during pregnancy is well established.

3) The main part of the circulating blood iron, the haemoglobin of the red blood cells, was long considered an important source for the foetal iron. It was thought that the erythrocytes were haemolysed and phagocytically destroyed by the foetal trophoblast. Further disintegration of the haemoglobin molecule was supposed to render the iron available for transmission to the foetus (Sholten and Veit, 1903; Hofbauer, 1905; Marshall, 1932; Stander, 1941). This theory was supported by the finding of degenerated red blood cells in the placental spaces (Sobotta, 1911; Asai, 1914; and others) and by the histochemical detection of iron in foetal and maternal structures of the placenta (Chipman, 1902; Hofbauer, 1905; Kropp, 1940; Dempsey and Wislocki, 1944). Sobotta, and with him Asai, suggested that the intact haemoglobin molecule is absorbed by the visceral yolk sac epithelium in mice in early stages of pregnancy. Wislocki and coll., 1945, 1946, in histochemical investigation of the endometrium and placenta of rodents, noted the presence of iron in the uterine glands and in the visceral yolk sac epithelium. They suggested that iron is absorbed by this epithelium from stagnant blood material in the degenerating decidua capsularis in the early stages of gestation, and later from the uterine contents enriched with iron from the uterine glands.

The work of Pommerenke, Hahn, Bale, and Balfour, 1942, threw new light on this problem. They administered radioiron to

pregnant women and were able to trace it in the foetal plasma after less than one hour, and in the foetal red blood cells after two hours in two of the reported cases. This speedy passage of the administered iron from the intestine of the mother to the foetal serum and red blood cells induced them to suggest that "at least some iron reaching the foetus does not have as its immediate precursor the iron contained in the haemoglobin of the maternal red blood cells." From experiments with radioactive iron in guinea pigs, Vosburgh and Flexner, 1950, concluded that the iron supplied to the foetus from the maternal plasma in the latter half of gestation is sufficient to account for the iron retained by the foetus during growth. They stress, however, that "the histological picture in very early stages of gestation gives clear and abundant evidence of disintegration of the red cells in the sinuses and villi of placenta, and until there is evidence to the contrary it must be assumed that maternal red cells may be an important source of fetal iron at these very early stages." *It is thus well established that in the latter half of pregnancy the foetal iron comes from the maternal plasma, to which it is delivered from the intestinal absorption and from the iron depots.*

Very little is known about the mechanism of this transfer, however.

Some interesting features of the iron transfer. — The serum iron concentration of the blood of the umbilical vein has been determined as about 160 γ % at term (Locke and coll., 1932; Thoenes and Aschaffenburg, 1934; Neuweiler, 1938; Vahlquist, 1940, 1941; Widmer, 1942; Black and Stoker, 1946; Laurell, 1947). Neuweiler found the serum iron of blood from the umbilical vein to be at a higher concentration (155 γ %) than the corresponding value for blood obtained from the umbilical artery (138 γ %) (The variation of the difference is very pronounced, however, as pointed out by Vahlquist, 1941.)

Vahlquist, 1941, determined the serum iron concentration in human foetuses from the 5th month of pregnancy to full term. He states that the values for the 5th and 6th months are fairly low, lower than the corresponding values for the maternal serum. But in the 9th and 10th months the serum iron level of the foetus increased to the level for the newborn infant, definitely above the serum iron level of the mother. Comparing the serum iron concentrations of the venous and arterial umbilical cord blood, Vahlquist was not able to detect differences reported by Neuweiler and Widmer. He points out

(Vahlquist, 1944) that the difference to be expected would hardly be measurable, the maximum calculated iron consumption of the foetus being 4 mg daily. Laurell, 1947, found the saturation limit of foetal serum at term to be about 226 γ %, that is, half the value obtained from maternal serum (446 γ %). Thus it can be established that the maternal serum iron concentration during the latter half of gestation decreases to subnormal values (about 60—80 γ %), whereas the serum iron level in the foetus during the same period rises to a value about twice as high (160 γ %). On the other hand, the total iron-binding capacity of the maternal serum rises from normal values during the first half of gestation to a maximum (450 γ %) in the last two months of pregnancy. The corresponding value for the foetal serum at term is only half as high (about 250 γ %). An explanation of the physiological significance of the different serum iron concentrations and total iron-binding capacities of maternal and foetal sera has been put forward by Laurell, 1947. The low serum iron concentration in the maternal organism during the latter half of gestation is accompanied by a high latent iron-binding capacity of the serum i.e. a lowering of the ratio, manifest iron-binding capacity of the serum: latent ditto, bringing about increased iron absorption from the intestine and mobilisation of available depot iron. The reverse conditions are found in the foetal organism. Here the ratio is high, giving rise to increased storage of iron in depot organs.

Laurell evaluates the different concentrations of iron-binding protein in maternal and foetal sera and concludes: "this goes against the view that the iron-binding protein component of the serum might pass freely through the placenta."

Thus, there seems to be no doubt that serum iron is the main source of foetal iron during the latter half of pregnancy, when the greater part of the foetal iron is transferred to the conceptus. But how this transfer is accomplished is not yet known. The passage of iron obviously goes against a concentration gradient, from the maternal serum poor in iron, to the foetal serum with a concentration twice as high. Furthermore, the concentration of the iron-binding protein in the two sera differs in the opposite direction.

Thus, a simple diffusion of the serum iron as such seems to be out of the question. Experiments on placental permeability in different species carried out with radioactive sodium, water, and inorganic phosphate (Flexner and coll., 1941—48) reveal that the rate at

which these compounds cross a unit weight of placenta is associated with a thinning of the barrier between the maternal and foetal circulations and increased area of exchange, and in full agreement with the laws of diffusion. In their corresponding investigation of the guinea pig, using radioiron, VOSBURGH and FLEXNER, 1950, found iron to behave quite differently. They observed no correlation to duration of pregnancy in the rate at which iron crossed the placental barrier at different stages of the latter half of gestation. These authors felt there was reason to postulate: "a rather complex regulatory mechanism which may be analogous to that concerned with the absorption of iron from the gastrointestinal tract."

CHAPTER II

The Problem

The preceding sections have shown that the problem of placental iron transfer from mother to foetus is far from settled. As a first step, it has been well established that the maternal serum iron is the source of foetal iron, with the possible exception of the earliest stages of pregnancy. The mechanism of its transfer across the placental barrier from the maternal to the foetal circulation is still disputed, however.

In view of the concentrations of serum iron and the iron-binding protein of the serum in maternal and foetal blood, a simple diffusion is considered out of question, which view is further supported by the fact that the iron-binding protein of the serum does not pass freely across the placental structure. As the maternal serum iron complex thus seems to be only the source of foetal iron but not the actual transferring principle, its iron component must be split off in some way. Whether the iron is liberated as ionised iron or temporarily exists in combination with some intermediate vehicle before appearing once more in the foetal serum as the serum iron complex is not known. This brings us to the following problems.

1. Is it possible to demonstrate histochemically the presence of some tissue iron compound in the placental structures?

2. If so, might this iron be associated with the transfer across the placenta, and is it possible to discover anything about its chemical nature?

3. Are there any quantitative variations of this hypothetical placental iron compound with advancing gestation or under different experimental conditions of the maternal animal, — variations offering the possibility of some conclusions as to the iron transfer mechanism?

CHAPTER III

Choice of the experimental animal

When dealing with problems of placental transfer, it is of importance to select an experimental animal with a placental organ as far as possible like that of man. GROSSER, 1927, on the basis of the number of tissue layers interposed between maternal and foetal circulation, classified placentas into four principally different types: the epithelio-chorial, syndesmo-chorial, endothelio-chorial and haemo-chorial type of placenta. The cellular layers between the two circulation systems in this classification of Grosser's are evident from Table I.

TABLE I.

Cellular layers between maternal and foetal circulation. Grosser's classification of placentas (after FLEXNER and GELLHORN, 1942).

Type of placenta	Maternal			Foetal			Examples
	Endo the-lium	Connec-tive tissue	Epi-the-lium	Epi-the-lium	Connec-tive tissue	Endo-the-lium	
Epithelio-chorial	+	+	+	+	+	+	Sow
Syndesmo-chorial	+	+	−	+	+	+	Goat, sheep, cow
Endothelio-chorial	+	−	−	+	+	+	Cat
Haemo-chorial	−	−	−	+	+	+	Guinea pig, rat, rabbit, man

It will be seen that the rodents usually encountered as experimental animals, i.e. the guinea pig, the rabbit and the rat, represent the haemo-chorial type of placenta, as does man. However, the placental organs of rodents differ in many respects from that of man, as will be pointed out more in detail later on. In view of the ease with which the sexual cycle of the rat may be determined, and the possibility of establishing the gestational age in a fairly precise manner, the rat was chosen as experimental animal. Other advantages are the short gestational period of this animal, and the rather well documented features of its iron metabolism.

CHAPTER IV

Morphological and physiological features of the rat placenta

The development and structure of the rat placenta has been the subject of thorough investigation. A fairly representative review of the more important articles is given by Bridgeman, 1948.

In discussing the placental structures in the rat, it is necessary to realize that besides the *allantoic* placenta — the only human placental organ — the *yolk sac* is also of considerable importance in this respect. Sobotta, 1911, suggested that the yolk sac absorbs maternal haemoglobin. Asai, 1914, was of the same opinion, and further stated that fat and glycogen are absorbed by this route. Brunschwig, 1927, considered the yolk sac to be the principal organ of iron salt transmission to the very young rat embryo. Everett, 1933 and 1935, stressed the placental function of the yolk sac in the rat and introduced the term *yolk sac placenta*. Wislocki and coll., 1946, considered that the visceral yolk sac epithelium absorbs iron and secretion products of the uterine cavity. They also suggest that this epithelium functions under anaerobic conditions, as it has a high glycogen concentration. Hard, 1946, concluded from the presence in this epithelium of alkaline phosphatases in the guinea pig that the yolk sac epithelium absorbs fat and sugar. Noer and Mossman, 1947, on the basis of surgical ligation of allantoic and vitelline vessels, stated that the yolk sac has an important placental function, which is complementary rather than supplementary. Bøe, 1951, studied the vascularisation of the yolk sac in the rat, and concluded that "the pattern of the vitelline circu-

lation points to the dominating importance of the villi area as an accessory nutritive organ for the fetus." It must therefore be considered as well established that the yolk sac is an important part of the placental organ in the rat.

In man the yolk sac disappears early in development and persists as a rudimentary sac through pregnancy, and can be found on the foetal placental surface between the chorion and amnion at term. At the beginning of pregnancy, however, it is a very prominent organ, and the largest structure connected with the embryo. It is later taken up by the embryo to form part of the intestinal canal and becomes partly enclosed by the abdominal wall, through which it protrudes from the umbilicus as a stalked sac, the stalk becoming narrower and longer as pregnancy advances. STANDER, 1941, pointed out that "it must however play an important part in the economy of the human embryo, as it develops considerable circulation."

In the rat the yolk sac becomes inverted by the embryonic ectoderm to form a narrow cavity, the *yolk sac cavity*. It is limited peripherally by the *parietal yolk sac wall*, see Fig. 1 which lines the foetal surface of the placenta and the uterine decidua. The inner wall of the inverted yolk sac, the *visceral yolk sac wall*, encloses the foetus and the amniotic cavity and meets the peripheral at its attachment to the foetal surface of the placenta, some distance from the insertion of the umbilical cord. The yolk sac cavity initially communicates with the mid gut through the vitelline stalk. This communication is obliterated in the middle of gestation, however, and the stalk from then on contains only the vitelline vessels, which isolates the circulation of the yolk sac from that of the allantoic placenta. The peripheral yolk sac wall is a homogeneous membrane, *Reichert's membrane*, see Fig. 1, to which a single layer of thin epithelium is attached. On the 16th day of gestation the parietal yolk sac wall ruptures, together with the overlying decidua capsularis, opening the yolk sac cavity to the uterine cavity. The visceral yolk sac wall is made up of two different parts, *the villous part* and *the circumference*. The villous part has a high columnar epithelium resting on a loose mesenchyma containing abundant vessels. The epithelium is folded in a complicated way, resembling intestinal villous epithelium. This villous part is situated in the vicinity of the foetal surface of the placenta, forming a collar-shaped structure, (see Fig. 1).

From a physiological point of view this part of the visceral yolk sac wall is considered to be of greater importance. The circumferential

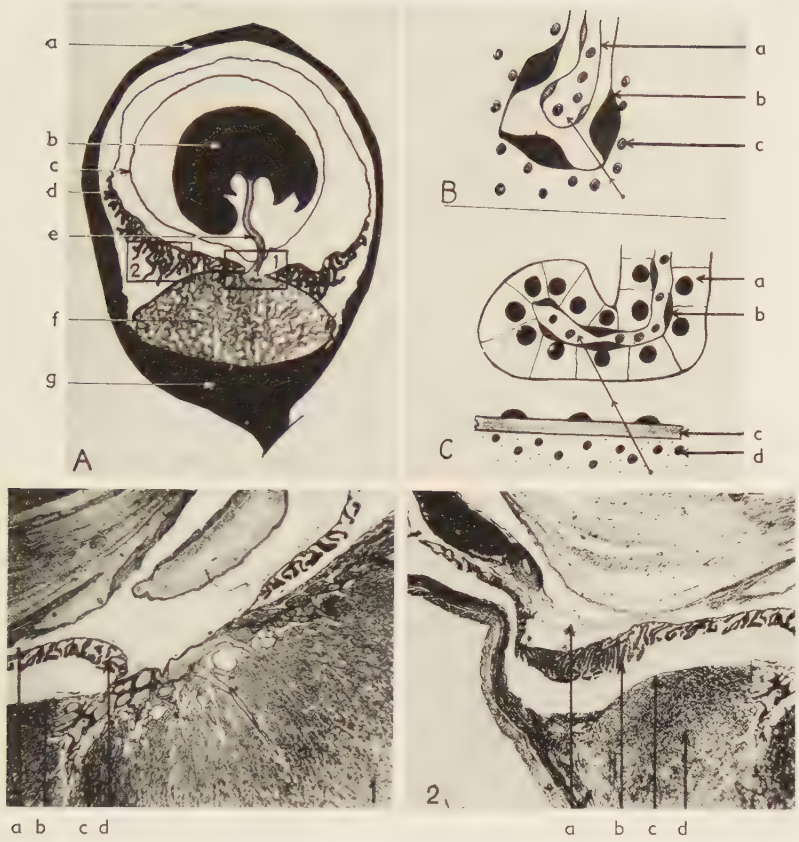


Fig. 1: *A*. Cross section of the pregnant rat uterus near term. a. Peripheral uterine wall. b. Foetus. c. Amnion. d. Visceral yolk sac wall at the beginning of the villous part. e. Umbilical cord. f. Allantoic placenta. g. Mesometrial part of uterine wall. *1*. Photomicrograph of the region of the attachment of the umbilical cord to the allantoic placenta (Enlargement: × 43). a. Amnion. b. Parietal yolk sac wall. c. Villous part of visceral yolk sac wall. *2*. Photomicrograph of the margin of the allantoic placenta. (Enlargement: × 43). a. Amnion. b. Villous part of visceral yolk sac wall. c. Parietal yolk sac wall. d. Labyrinthine pattern of allantoic placenta.
B. Route of transfer through a chorionic villus (indicated by the oblique arrow). a. Capillary endothelium. b. Syncytium. c. Maternal blood.
C. Route of transfer through the yolk sac placenta (indicated by the oblique arrow). a. Visceral yolk sac epithelium. b. Capillary endothelium. c. Parietal yolk sac wall (including Reichert's membrane). d. Maternal blood.

portion has a cuboidal epithelium and does not exhibit the villous formations described, see Fig. 1.

The allantoic placenta of the rat — like that of man — is of the

haemochorial type, acc. to GROSSER, 1927, i.e. the chorionic tissue is bathed directly in maternal blood. During placentation, maternal blood vessels are penetrated by trophoblastic cells. The allantoic vessels covered with a sheath of syncytial cells form an intricate labyrinthine pattern within the maternal blood sinuses. So in early gestation chorionic villi are covered with a single layer of syncytial cells. In later pregnancy, however, this layer thins out to such a large extent that MOSSMAN, 1926, has suggested the term "haemo-endothelial" for the rat placenta at this period. We must therefore consider two different routes for placental transfer in the rat, see Fig. 1.

1) Substances using the allantoic placenta reach the foetal circulation from the blood in the maternal blood sinuses of the allantoic placenta, crossing the syncytial and endothelial layers. 2) Substances using the yolk sac placenta leave the maternal blood in the sinuses underlying the foetal surface of the placenta, pass through the parietal yolk sac wall, and after being absorbed by the epithelial cells of the villous part, they reach the foetal circulation via the vitelline vessels.

Another transfer route was stressed by WISLOCKI and coll., 1945—46. They pointed out that after the rupture of the parietal yolk sac wall on the 16th day of gestation the visceral yolk sac epithelium is bathed by uterine secretions. They suggested that the absorption of these secretions contributes to the nutrition of the foetus, especially with regard to iron. This has been disputed by NYLANDER, 1951 and CAMPBELL and NYLANDER, 1952.

CHAPTER V

Histological and histochemical examinations of the rat placenta with special reference to iron, and the nature of Reichert's membrane

A. The test animals

These correspond in every respect with those used in the chemical part of this study (for details see Chapter VII). They were divided into two main groups of 'normal' and 'anaemic' pregnancies, each

of which included animals on the 15th, 17th, 19th, 20th and 21st days of pregnancy. The animals included in the anaemic pregnancy group were rendered anaemic by daily bleeding, which produced a haemorrhagic anaemia well established and constant during the experimental period. The technique used to induce the experimental anaemia is described in detail in Chapter VII. That chapter also deals with some possible drawbacks with this form of experimental anaemia. A total number of 20 animals was used, equally distributed in the two main groups and the different days of gestation.

B. Experimental technique

The operative instruments were of stainless steel and as the glassware carefully cleaned to ensure they were iron-free. The chemical reagents were of analytical purity.

Under light ether anaesthesia the abdomen was opened, the uterine branches exposed, — though remaining connected to the animal, — and every second uterine swelling, representing one egg, incised. The visceral yolk sac membrane was isolated, cut out and unfolded upon a glass slide. The fresh specimen thus obtained was incubated for 10 min. in a solution prepared previously, consisting of equal parts of 20 per cent iron-free hydrochloric acid and 10 per cent aqueous solution of potassium ferrocyanide. Before use this solution was of a bright yellow colour, completely free from the blue or greenish tint of Prussian blue.

The rest of the eggs were cut out and immediately fixed in a cold mixture of absolute methanol and absolute ethanol. Finally, from some animals not included in those mentioned above — some from the anaemic pregnancy group and the others from the normal, all on the 20th day of gestation — some eggs were removed, the visceral yolk sac cut out and unfolded as already described. The allantoic placenta was moulded between two glass slides to obtain a thin, flattened specimen. These slides were then incubated with a few drops of 10 per cent aqueous solution of cadmium sulphate in a damp chamber at 37° for 24 hours acc. to Granick, 1946.

The *fixed material* was embedded in paraffin wax in vacuo and cut with a microtome. The sections were stained for 1. iron, 2. elastic fibres, 3. collagenous structures, 4. Schiff-positive substances, as follows.

1. Several of the different iron reagents of histochemistry were tried, and the Tirmann-Schmelzer modification of the Turnbull blue reaction was found to give the most constant and intense reactions.

The *fresh specimens* incubated in hydrochloric acid potassium ferrocyanide solution did produce a pronounced Prussian blue colour. This colour was partly soluble in all the fixing liquids used and because of this the specimens were not used in histological technique.

2. The elastic fibres were stained with resorcin fuchsin according to WEIGERT's method. (ROMEIS, 1948.)

3. Collagenous structures were demonstrated by means of Heidenhain's 'pikroblauschwarz'. (ROMEIS, 1948.)

4. The periodic acid-leucofuchsin technique of HOTCHKISS, 1948, was used in staining Schiff-positive elements of the tissues. (GLICK, 1949.)

C. Results

1. *Iron*

a. The Prussian blue reaction in fresh specimens of the visceral yolk sac membrane

The visceral yolk sac membranes of the *normal pregnancy group* showed an intense blue colour after 10 min. incubation in the hydrochloric acid potassium ferrocyanide solution. This colour reaction was strictly limited to a circular margin in the close vicinity of the attachment of the membrane to the umbilical cord (in the photographs represented by the central perforation) see Plate I. The Prussian blue colour gradually faded out towards the periphery, there being no colour at all in the outermost part. The circular region developing the colour was shown to be identical with the villous portion of the membrane, the uncoloured peripheral part with the circumference.

In order to ascertain that the Prussian blue reaction was not caused by the haemoglobin iron of the rather large blood content of the visceral yolk sac membrane, and its villous portion in particular, the specimens were examined microscopically. The red blood cells were of the dark brownish colour of acid haematin, and no blue or greenish tint was observable over the blood vessels. The Prussian blue colour seemed to be localised to the epithelial cells of the villi. In the *anaemic pregnancy group* this colour reaction was much less pronounced, and in some cases quite absent (see Plate I). *The villous part of the*

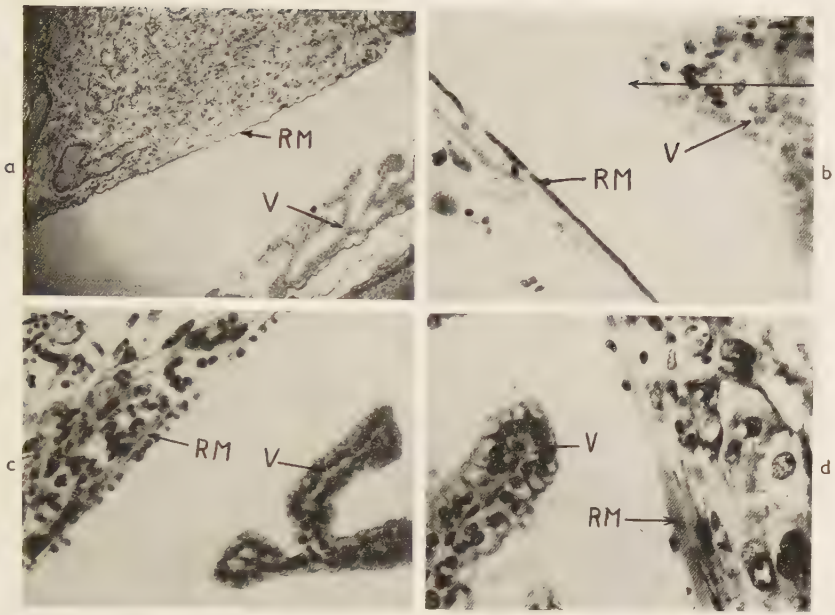


Fig. 2. Turnbull blue reaction in placental tissues (Tirmann-Schmelzer; counterstain: 'Kernechtrot'). Reichert's membrane (RM) shows clearly positive iron reaction in the sections obtained from normal pregnancy (a and b). The visceral yolk sac epithelium (V) contains blue granules of iron-precipitate in the supranuclear regions (indicated by the arrow) hardly visible in the high power view. c and d.: The corresponding sections from anaemic pregnancy reveal a very faint iron-reaction in Reichert's membrane (RM), and no iron-positive granules are seen in the visceral yolk sac epithelium (V). (Enlargement: a. × 76. b. and 3.: × 325. c. 4.: × 760.)

visceral yolk sac membrane in the normally pregnant rats was thus shown to contain ferric iron which reacted with potassium ferrocyanide in acid solution, and which did not belong to the haemoglobin. The animals of the anaemic pregnancy group were shown to contain much less ferric iron reacting in this way.

b. The Turnbull blue reaction in histological sections of the rat placenta

The results of this reaction were in accordance with the findings described in a preliminary report (Nylander, 1951). Thus in *normal pregnancy* iron was demonstrated in the villous portion of the visceral yolk sac membrane as blue granulations or as a bluish tint of the supranuclear regions of the epithelial cells. Furthermore, the Reichert membrane was intensely blue throughout, and stood out sharply

against the surrounding structures, see Fig. 2. No iron was found in the allantoic placenta in the stages of pregnancy examined by this method. In the *anaemic pregnancy* group no iron was found under the same conditions. The Reichert membrane was unstained or showed only a slight greenish tint in a few sections. *The villous portion of the visceral yolk sac membrane in normally pregnant rats was shown to contain iron, demonstrable as ferrous ferricyanide after reduction with yellow ammonium sulphide by the Tirmann-Schmelzer method. Compared with the Prussian blue reaction of the fresh specimens, the iron reaction obtained was poor. The Reichert membrane gave a strongly positive iron reaction. In the anaemic pregnancy group no iron, or very little, was found in the corresponding tissues. The allantoic placenta of both the normal and the anaemic pregnancies contained no iron demonstrable with this technique.*

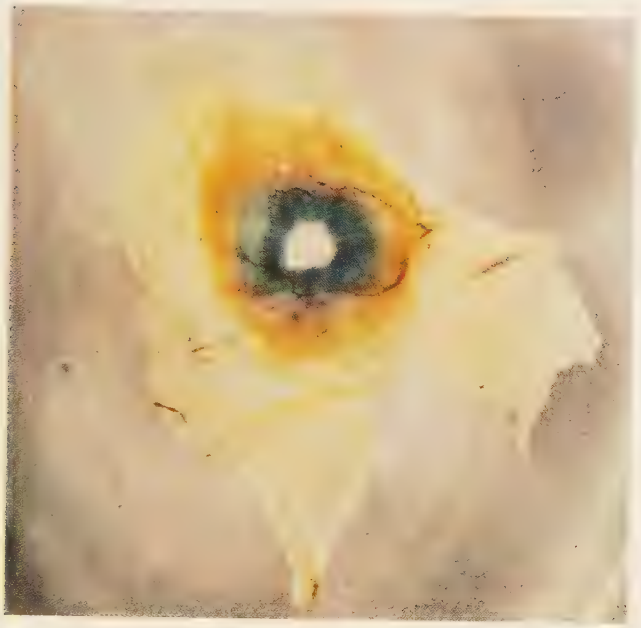
c. The ferritin content of the rat placenta

In the fresh specimens of the visceral yolk sac membrane and the allantoic placenta incubated in cadmium sulphate, ferritin was observed as typical octahedral, yellowish-brown crystals. *In normal pregnancy* the crystals were abundant in the villous portion of the visceral yolk sac, but there were no crystals in the peripheral part of the membrane corresponding to the circumference. The number of ferritin crystals in the fresh specimens of the allantoic placenta was generally less, see Fig. 3. The polarisation microscope showed the ferritin crystals to be isotropic, as pointed out by Granick, 1942.

In the *anaemic pregnancy group* no ferritin crystals — or very few — were found in the yolk sac and allantoic placenta specimens. *Thus, in the normal pregnancy group a rather high ferritin content, revealed as ferritin crystals on incubation with cadmium sulphate, was observed to exist in the villous part of the visceral yolk sac membrane, and to a lesser degree in the allantoic placenta. In the corresponding tissues of the anaemic pregnancy group, very little ferritin was demonstrable by this method.*

d. Discussion

It has been shown that the villous portion of the visceral yolk sac wall contains iron in a form demonstrable by incubating the *fresh specimen* in hydrochloric acid potassium ferrocyanide solution. The Prussian



1.

2.

1. Iron-positive reaction (Berlin blue) in the fresh yolk sac membrane of normal pregnancy.

2. Absence of any iron-positive reaction in the fresh yolk sac membrane of anaemic pregnancy.

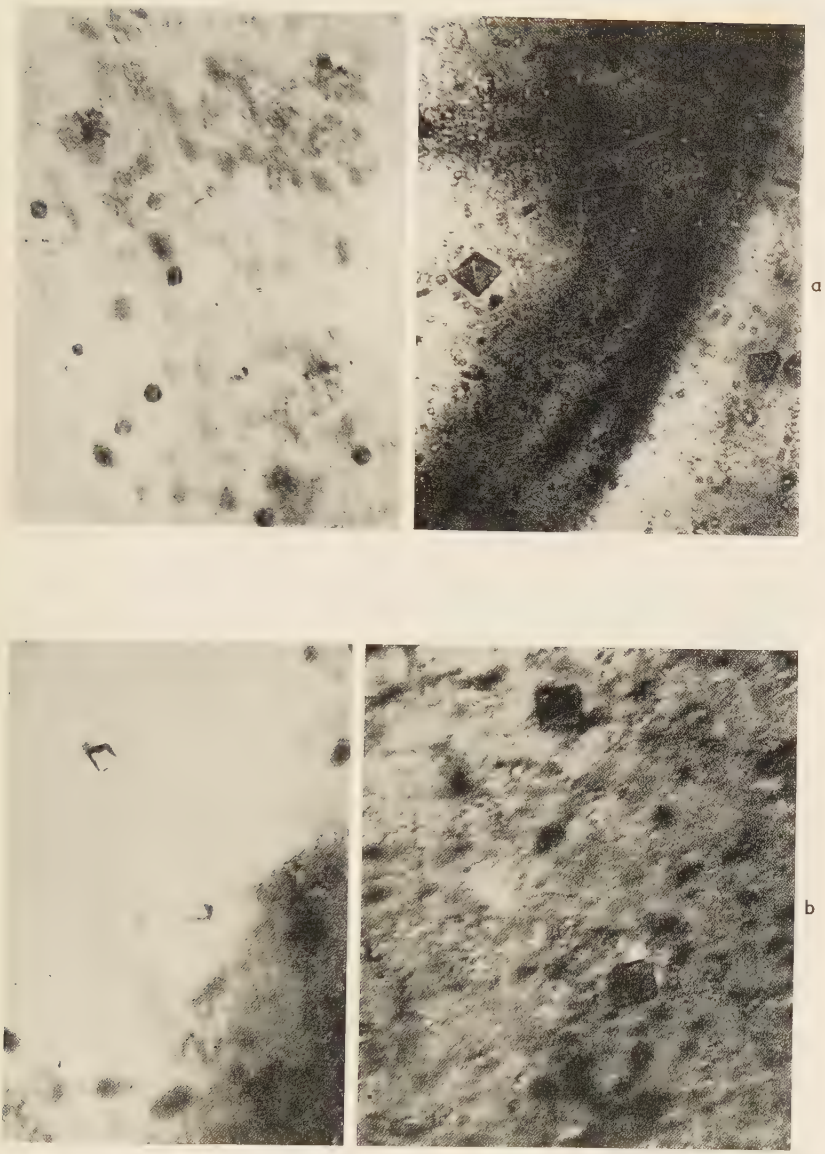


Fig. 3. a. Ferritin crystals in fresh tissue of the visceral yolk sac membrane. The large numbers of crystals are seen in the first plate and, in the high power view (right), the typical octahedral form is seen in the margin of an air bubble. (Enlargement: × 176 and × 325 resp.) b. Ferritin crystals in fresh specimen of allantoic placenta. (Enlargement: as in a.)

blue reaction obtained is very intense in the normally pregnant material. Staining of the *histological sections* produces a positive Turnbull blue reaction of a much lower intensity. This might be caused by a loss of iron in the histological treatment, or alternatively most of the iron demonstrated by the Prussian blue reaction of the fresh specimens might exist in another chemical composition than that reacting in the histological sections.

It has been shown that ferritin is present in considerable amounts in the villous portion of the visceral yolk sac membrane in normal pregnancy. It is generally thought that ferritin is not demonstrable in histological sections, only haemosiderin having this property. However, the localisation and intensity of the Prussian blue reaction of the fresh specimens is in conformity with the appreciable amounts of ferritin demonstrable in the same region of the visceral yolk sac membrane. It must therefore be considered justifiable to assume that the Prussian blue reaction mainly is due to the ferritin iron of the yolk sac membrane. The Turnbull blue reaction for histological sections of the corresponding tissue might be due to non-ferritin iron, representing iron in excess and histochemically demonstrable as haemosiderin. Furthermore, the ordinary histochemical technique revealed no iron in the allantoic placenta of the histological sections, though the allantoic placenta had been shown to contain ferritin.

To sum up, the villous portion of the visceral yolk sac membrane in normally pregnant rats contains iron demonstrable as Prussian blue precipitates in the fresh specimens, this iron fraction being only poorly demonstrable with the ordinary histochemical technique. The distribution of this iron corresponds closely to that of ferritin, and it is supposed that it is mainly ferritin iron. The iron demonstrated by the ordinary histochemical technique applied to the tissue sections is supposed to represent another iron fraction, presumably haemosiderin. Furthermore, ferritin is present in the tissue of the allantoic placenta in normal pregnancy, but the ordinary histochemical technique revealed no iron. In anaemic pregnancy, all the above tests for iron and ferritin gave results which were either negative or only slightly positive.

Notes on some track autoradiographic studies of the rat placenta

Campbell and Nylander, 1951, showed by means of a track autoradiographic technique that in normal pregnancy injected radioactive iron accumulates in considerable quantities in the visceral yolk sac membrane of the rat.

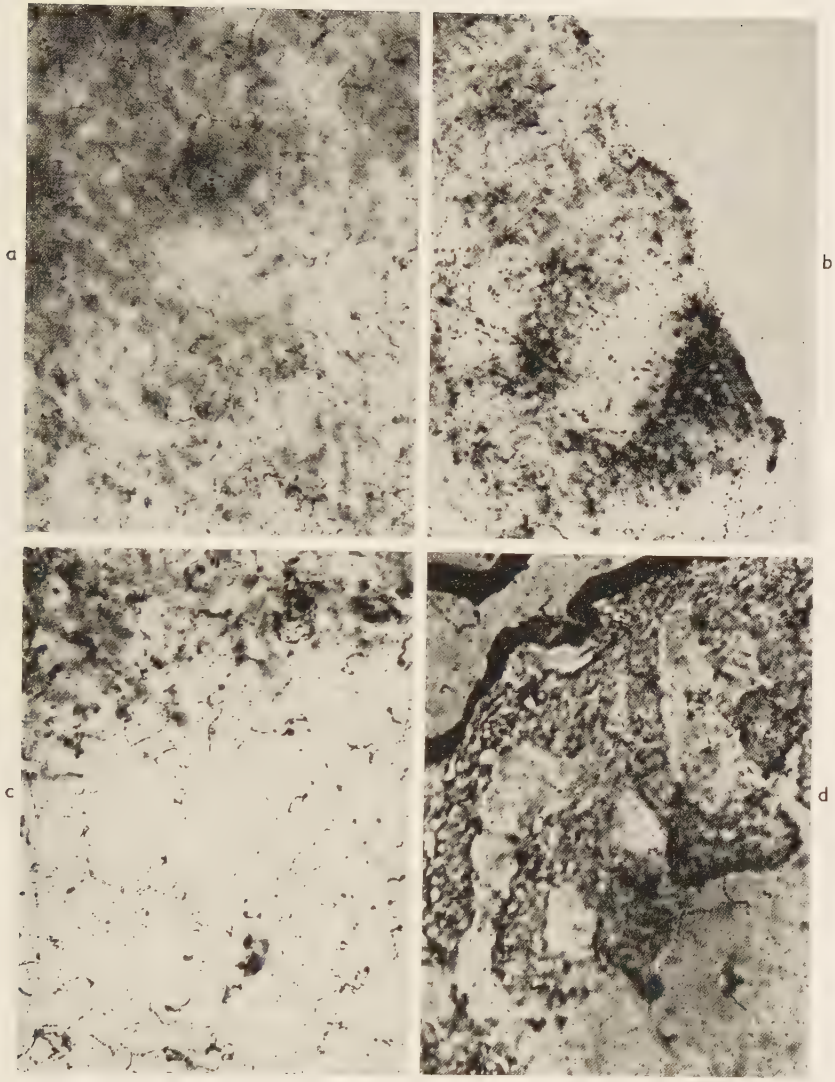


Fig. 4. Autoradiographs of allantoic and yolk sac placental tissues in normal and anaemic pregnancy. (Enlargement: × 325.) a. Allantoic placenta of normal pregnancy. b. Yolk sac placenta of normal pregnancy. c. Allantoic placenta of anaemic pregnancy. d. Yolk sac placenta of anaemic pregnancy.

This was considered to bear out the important rôle of the yolk sac placenta in the iron transfer process. This is in good agreement with the histochemical result that this structure contains rather abundant iron, and lends strong

support to the assumption that this iron represents an essential part of the placental iron transfer from mother to foetus.

In connection with some experiments with radioactive iron, to be referred to in a later chapter, the autoradiographic method was applied to specimens taken from normally and anaemically pregnant rats (for technical details see page 89 and Campbell and Nylander, 1951).

The results of this investigation, including semi-quantitative determinations of the activity in the structures concerned will be given later by Cambpell and Nylander.)

Only some representative autoradiograms will be considered here (see Fig. 4). It will be seen that the track frequency is rather high in the visceral yolk sac membrane of the normal animal, and considerably less in the anaemic one. The allantoic structures of both the normal and anaemic animals have about the same track frequency. However, the allantoic placentas were not freed from blood, and activity arising from this might interfere with the results.

But the results *do* confirm a feature of the iron demonstrated histochemically — the diminution in quantity when the mother has a deficiency of iron — as far as the injected radioactive iron accumulated in the visceral yolk sac wall is concerned. The quantitative determinations of the radioactivity from the structures concerned are given in Chapter X.

It has been pointed out that Reichert's membrane gave a positive iron reaction in the histological specimens from all stages of normal pregnancy examined. This might be due to a high iron content of the yolk sac cavity lining the membrane. However, the fact that the membrane was uniformly penetrated by the blue colour makes this explanation less probable. Alternatively, it might be a false reaction, not indicating the presence of iron; but the reaction is absent or faint in the sections from the anaemic pregnancy group. The fact that the blue colour does not appear in the iron-deficient rats supports the view that it is a characteristic of iron.

It is therefore not impossible that Reichert's membrane contains some loosely bound iron and that this iron may be involved in the iron transfer.

2. *The structure of Reichert's membrane*

Wislocki and coll., 1946, stated that this membrane consists of compacted collagen, possessing intense affinity for acid dyes, strong parallel birefringence, bluish white fluorescence and lack of affinity for silver. Everett, 1935, noted that it takes a faint bluish tint following intravital injections of trypan blue.

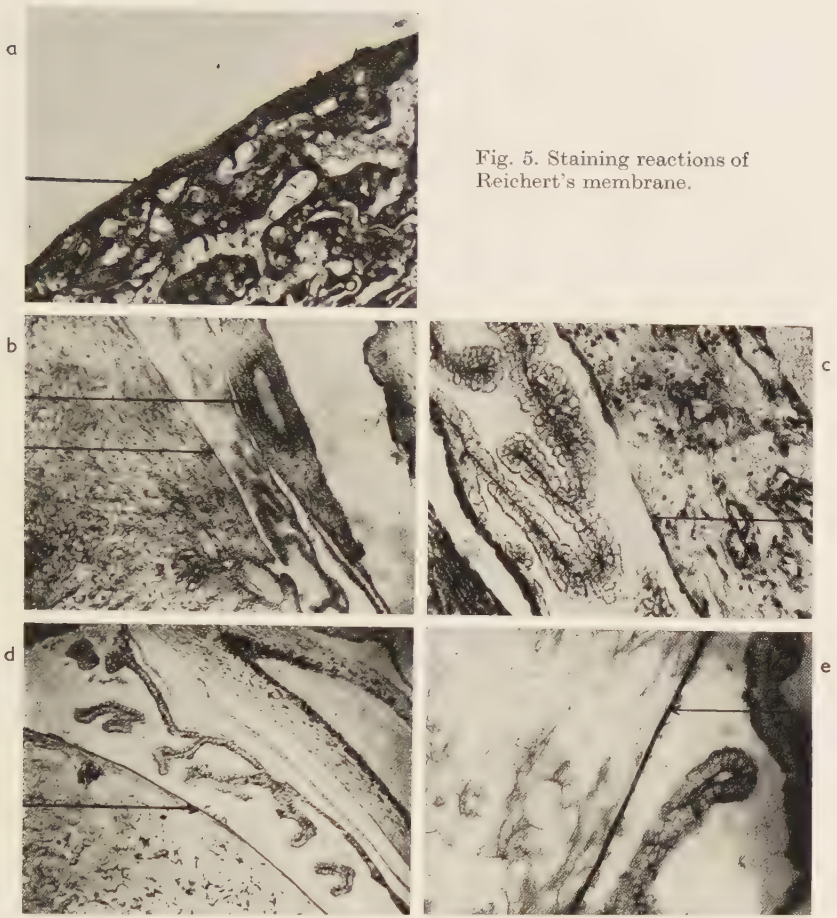


Fig. 5. Staining reactions of Reichert's membrane.

a. The collagenous structure of Reichert's membrane (indicated by the arrow) is clearly seen. (Heidenhain's 'Pikroblauschwartz' stain. Enlargement: × 760.) b and c.: The elastic elements of the foetal vessel stand out sharply (indicated by the first arrow in fig. b). Reichert's membrane (the second arrow in fig. b.) does not appear to contain any elastic elements. (Weigert's elastic stain; Enlargement: × 76 and 325, respectively.) d. and e. The Hotchkiss positive staining reaction of Reichert's membrane is indicated by the arrow. (Hotchkiss acid leucofuchsin. (Enlargement: × 76 and 325, respectively).

Staining reactions obtained

1. With Weigert's resorcin fuchsin, Reichert's membrane did not give the staining reaction of elastic elements, and this applied to all the membranes examined. The elastic fibres of the arterial walls as

well as the elastic cartilaginous structures of the foetus were intensely stained in all these sections, see Fig. 5. *These results thus bear out the statement that Reichert's membrane does not contain elastic elements.*

2. In staining the collagenous fibres with Heidenhain's 'pikroblauschwartz', Reichert's membrane turned intensely black in every section examined, indicating their collagenous nature, see Fig. 5. *The statement of Wislocki and coll. that the membrane contains collagenous fibres was thus verified.*

3. When Hotchkiss's periodic acid-leucofuchsin method was applied to the sections, it was noted that the actual membrane appeared strongly red throughout the material examined. This reaction was proved to be independent of the presence of glycogen.

Reichert's membrane therefore also contained some Schiffpositive substances which might be mucopolysaccharides. This conjecture is supported by the findings of Everett, 1935, who noted that intravitally injected trypan blue stains the membrane and also appears in the next foetal layer, the visceral yolk sac epithelium, indicating a passage through the membrane. According to Gersh & Catchpole, 1949, the uptake of the stain in basal membranes conforms with the assumption that glycoproteins form an integral part of such structures. *Thus Reichert's membrane might be justifiably described as a permeable structure consisting of collagen and some polysaccharide material.*

CHAPTER VI

Chemical determinations

It was shown in the preceding chapter that both the placental organs of the rat contain iron compounds considered to be involved in the placental transfer of iron, and demonstrable by histochemical methods.

This and following chapters deal with determinations of some foetal and placental iron fractions, which were undertaken to investigate whether the quantitative variations provide any information about the transfer process.

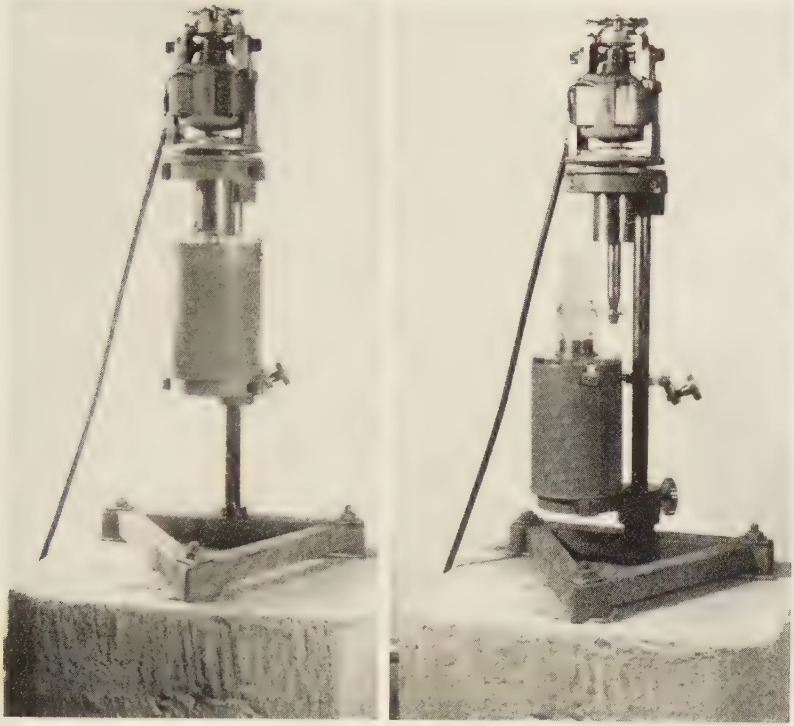


Fig. 6. Homogeniser, for description see text.

1. Determinations of the hydrolysable iron in tissues. (The Method 1)

a. ***Principle***

Tissues to be analysed for the amount of iron hydrolysable under certain standard conditions were homogenised to give the same distribution of animal material in all the analyses performed. The final analysis was thus carried out on the same amount of biological material in every case. A given volume of the homogeneous suspension obtained was hydrolysed with hydrochloric acid, which dissolves the hydrolysable, acid-soluble iron of the tissues. The proteins were precipitated with trichloracetic acid, and when the pH was optimal, a reducing agent was added to the clear filtrate, and the iron then determined colorimetrically as ferrous o-phenanthroline complex.

b. *Apparatus*

1. *Homogeniser*, see Fig. 6. In order to obtain complete homogenisation of small quantities of organic material in small liquid volumes, a special homogeniser was constructed. The motor was of the same type as that of the commercial 'Turmix' blendor. The speed was adjustable by means of a rheostat, the maximum speed being about 14 000 r.p.m. The shaft had a stainless knife screwed to its lower end, the four blades of the knife being inclined to one another. The hopper, adjustable vertically, had a packing of hard rubber at the bottom into which a glass container fitted. The container was made of thick pyrex glass and had four ridges projecting from its inner surface, to prevent the material being homogenised from moving round outside the radius of the rotating knife. The glass containers were of two sizes, 10 and 25 ml.

2. *The commercial 'Turmix' rotary blendor* was used to deal with larger amounts of tissue.

3. *Beckman DU quartz spectrophotometer.* The cuvettes used were 1.000 ± 0.002 cm thick.

c. *Reagents*

1. *Hydrochloric acid* (LKB), iron-free, 20 %.
2. *Trichloracetic acid* (LKB), iron-free, 20 % aqueous solution.
3. *Paranitrophenol* (Merck), 1 % alcoholic solution.
4. *Ammonium hydroxide* pro analysi (Merck), about 20 %.
5. *Sulphuric acid* pro analysi (Baker), 1/2 n.
6. *Hydroquinone purum* (Merck), 2 % solution (0.2 gm hydroquinone purum was dissolved in 10 ml bidistilled water and some drops of sulphuric acid were added).
7. *o-Phenanthroline hydrochloride* (Merck), 1 % solution, acidified with some drops of sulphuric acid.
8. *Iron standard solution.* 7.01 gm ferric ammonium sulphate, 10 gm ammonium sulphate and 10 ml concentrated sulphuric acid were dissolved in bidistilled water and made up to 1 litre. The iron was determined gravimetrically after oxidation with hydrogen peroxide, and the iron concentration given therefore corresponds to the total iron in mg per ml. Six determinations gave a mean of 0.833 mg of iron per ml[1].

[1] This standard solution was put at my disposal by AB Pharmacia, Upsala.

d. *Cleaning of glassware and other utensils*

After washing in soap and water, all glass apparatus used stood in bichromate sulphuric acid overnight, and was then rinsed with running tap water for 48 hours, after which it was rinsed in bidistilled water and dried in a drier. The shaft and knife of the homogeniser were cleaned in tap water and bidistilled water, and finally dried with absolute alcohol and analytical ether.

e. *Procedure*

1. After weighing in closed weighing vessels, the organic material was homogenised in bidistilled water. *In all analyses, the amount of water used corresponded to 10 times the weight of tissue.* In this way a constant distribution of the organic material in the suspension was obtained in every analysis, provided the homogenisation was complete. This was accomplished by rather slow rotation of the knife for 1 min. to begin with. The speed of rotation was then gradually increased up to the maximum, and kept at this value for 5 minutes. The resulting product was a homogeneous suspension which did not leave a visible sediment after standing for 24 hours. Microscopic examination of the suspension did not reveal any cell structures. This was the case with both the homogenisors used.

2. 1 ml of the suspension was drawn off with a pipette into a volumetric flask with a ground stopper. 1 ml of hydrochloric acid was added, and the vessel stored in a thermostatic chamber at 37° for 48 hours with continuous shaking.

3. To the hydrolysate thus obtained were added 2 ml trichloracetic acid, and after vigorous shaking the mixture was filtered (Munktell 00) into a volumetric flask. The residue was repeatedly rinsed with small volumes of trichloracetic acid, and the filtrate made up to 5 ml.

4. 3 ml of the clear filtrate were pipetted off into another 5 ml volumetric flask, 2 drops of paranitrophenol were added, and titration performed with ammonium hydroxide until the appearance of the yellow indicator colour showed the solution to be neutral. Sulphuric acid was added until the yellow colour disappeared, after which another 4 drops were added. The titration was performed with microburettes.

5. 3 drops of the hydroquinone and o-phenanthroline solutions

were added, the volume made up to 5 ml and the solution allowed to stand for 1 hour before proceeding with the determination.

6. The determination was carried out with a Beckman DU quartz spectrophotometer using cuvettes 1 cm in depth. Readings were made against a blank consisting of all the reagents used in the analysis, passed through the whole analytical procedure. Furthermore, the extinction of the blank was always read against bidistilled water. The wave length used was 510 μμ.

f. *Calculation of the value*

The amount of hydrolysable iron in mg per 100 gm of tissue was obtained in accordance with the following formula:

$$\frac{E \times K \times 275}{30}$$

E represents the extinction reading and is corrected with respect to the blank.

K represents the constant obtained in the photometrical measurement.

g. *Accuracy of the method*

The accuracy of the method was checked by analysis of iron salts of different known concentrations. (The original iron standard solution already mentioned was diluted to 666 γ % iron, — 8 ml of the

Table II.

Determinations of iron in iron salt solutions.

Concentration of iron in γ %			
Calculated	Measured	Calculated	Measured
I: 333	332	II: 167	170
333	335	167	163
333	334	167	166
333	330	167	168
333	338	167	168
III: 83	87	IV: 42	43
83	83	42	42
83	82	42	43
83	85	42	41
83	83	42	41

Table III.

Determinations of iron in biological materials with added iron.

Amounts of iron in γ					
I		II		III	
Calculated	Measured	Calculated	Measured	Calculated	Measured
4.37	4.17	4.46	4.54	4.00	3.83
4.37	4.33	4.46	4.42	4.00	3.92
4.37	4.08	4.46	4.46	4.00	4.00
4.37	4.46	4.46	4.50	4.00	3.92
4.37	4.25	4.46	4.42	4.00	3.92

Table IV.

The error of the measurements (σ_m) in determinations carried out on different tissues used in the investigation.

Tissue examined	No. of double determinations	Mean mg per 100 gm wet weight	σ_m	σ_m per cent of mean
Foetus (n. p. g.)[1]	22	2.17	0.05	2.1
Foetus (a. p. g.)[2]	19	1.36	0.04	2.8
Allantoic placenta (n. p. g.)	22	2.04	0.05	2.6
Allantoic placenta (a. p. g.)	19	1.63	0.04	2.7
Maternal liver (n. p. g.)	22	9.29	0.20	2.2
Maternal liver (a. p. g.)	19	3.92	0.08	2.1

[1] n. p. g. = normal pregnancy group.
[2] a. p. g. = anaemic pregnancy group.

standard solution made up to 1 000 ml — and by further diluting the approximate concentrations 333, 167, 83 and 42 γ % were obtained.) The results of these determinations are given in Table II.

A further check was made by adding a known quantity of iron to the biological materials used in the investigation (3.33 γ of iron — i.e. 0.5 ml of the 666 γ % iron standard solution — were added to 0.5 ml of homogenised tissue, the iron content of which is given as the mean value of 5 analyses performed). The results of these determinations are shown in Table III.

The reproducibility of the method has been estimated by statistical calculations on a series of double determinations performed on the tissues analysed here. The results are presented in Table IV.

The error of the method is given as σ_m, which means that the real value of the concentration of iron hydrolysable under these conditions has a probability 0.68 of differing by at most σ_m from the value obtained in one single determination, a probability 0.95 of differing by at most 2 σ_m, and is practically always nearer than 3 σ_m. It may be seen from Table IV that the error of the method is approximately 2—3 % of the mean value observed. The method may therefore be accepted as giving a result of sufficient accuracy.

h. *Discussion of the principle of the method*

Two different fractions of tissue iron have previously been referred to, distinguishable from each other chemically and physiologically.

The chromoproteins. From the viewpoint of the method it is of importance whether the iron contained in these compounds is liberated by acid hydrolysis.

Barkan, 1927—1939, introduced the term 'easily split off' iron of the blood. There has been much discussion about this postulated fraction of blood iron. Barkan isolated this iron fraction by acid hydrolysis of whole blood using hydrochloric acid of varying concentrations (0.4—1.2 %; he finally chose the 1.2 % acid) and incubation at 37° for 24 hours. Quantitatively, it averages 2—3 mg %, which is some 6 % of the total iron of the blood. In later publications (1937—1939) Barkan states that this iron is to be considered as a product of the physiological destruction of the haemoglobin from aging red cells and called it 'pseudohemoglobin.' The availibility of its iron could be ascribed to an open porphyrine ring. The existence of this fraction in a biological sense and its proposed physiological significance have been critically disputed by a number of writers (Moore and coll., 1937—1939, Venndt, 1940, Miller and Hahn, 1940, Heilmeyer, 1944, Grinstein and Moore, 1949). The iron fraction liberated by acid hydrolysis of whole blood was found to vary in amount, depending upon the acid used and its concentration. Thus Moore and coll., 1937, showed that using hydrochloric acid the maximum yield occurred at a normality of 0.05, but stronger acid tended to give less iron in the hydrolysate. Experiments with labelled iron by Grinstein and Moore, 1949, definitely prooved 'easily split off' iron to be an artefact emanating from the intact haemoglobin.

Two basic methods of liberating the non-chromoprotein tissue iron are used in determining this fraction directly.

Starkenstein and Weden, 1928, were the first to employ acid hydrolysis of finely minced tissue. Since then variations of this method are met with in the literature, and they include the use of different acids, e.g. hydrochloric or acetic acids of varying concentrations, with or without boiling and incubation for different times. (Sherman, Elvehjelm, and Hart, 1934; Andersson, 1950; and others.) The proteins were usually precipitated by trichloracetic acid, thus

introducing another hydrolysing agents into the process. The iron was then determined in the clear, protein-free filtrate, usually as ferrous bipyridine or o-phenanthroline complex.

Tompsett, 1934, 1935, introduced reducing agents to split off the iron component of non-chromoprotein tissue iron. He was followed by others, and the reducing agents usually mentioned include sodium hydrosulphite, sodium pyrophosphate, and thioglycolic acid (Shackleton and McCance, 1936; Shorland and Wall, 1936; Borgen and Elvehjem, 1937; Lederer, Balliére, and Van Damme, 1946). As the proteins were precipitated ordinarily with trichloracetic acid, the hydrolysing effect of this agent was added to the reaction.

There does not seem to be any difference between the two methods as regards the quantity of iron liberated from the haemoglobin, and the figures given seem to amount to 5—10 % of the total blood iron.

Ramsay, 1951, using hydroxylamine hydrochloride as reducing agent, and precipitating the proteins with ethanol, writes: "The non-haem Fe method gives erroneous results when applied to whole blood or to any other material containing an unusually large excess of haem over non-haem Fe."

It seems reasonable to conclude from the literature cited that when the non-chromoprotein iron in tissues is determined by acid hydrolysis or by reducing agents, some of the haemoglobin iron fraction is liberated. The amount of this varies: sometimes it represents all of Barkan's 'easily split off' iron, sometimes it is only part of it, presumably depending upon the acid or reducing agent used, its concentration, the time and temperature used in incubation, etc.

Non-chromoprotein iron. This fraction, earlier referred to as the storage-iron fraction, consists, as far as we know at present, of ferritin and haemosiderin.

Laufberger, 1937, pointed out that the iron content of ferritin is dissolved as the ferric ion by diluted hydrochloric acid. Kuhn, Sörensen and Birkhofer, 1940, dialysed it against normal hydrochloric acid, and demonstrated the migration of iron into the ultrafiltrate. The ferritin iron is thus acid-soluble and dialysable. The latter investigators also showed ferritin to be very resistant to reducing agents. The transformation of the iron from the ferric to the ferrous state is accompanied by release of the iron component and denaturation of the proteid. As to haemosiderin, Cook, 1929, using a histochemical technique, demonstrated the solution of iron from haemosiderin granules when acid was added. As previously mentioned, the serum iron is acid-soluble and dialysable in the same way. This iron fraction is however of no importance in the determinations we

are considering, since its concentration is negligible compared with the storage-iron fraction in the tissues examined. The iron protein complexes comprising this fraction all lose their iron component on acid hydrolysis.

There are *three methods*, differing in principle, that may be applied to the determination of the storage-iron concentration in tissues.

1. The organs to be analysed may be emptied of their blood contents. This can be done by washing the isolated, whole or disintegrated organ (Wakeham and Halentz, 1936 and others), or more effectively by perfusion with physiological saline in vivo and/or post mortem (Whipple and coll. 1932, 1936; Austoni, Rabinovitch and Greenberg, 1940; Schairer and Rechenberger, 1944, 1948; Kinney, Hegsted and Finch, 1949, among others). The total amount of iron is then determined after wet or dry ashing of the organic matter and estimated as the desired fraction. It should be pointed out, however, that washing by itself and even thorough viviperfusion do not free from blood. Austoni and coll. did not obtain satisfactory results for certain organs, e.g. the testes, the bone marrow and deep muscles. Furthermore, these methods might not only wash out the blood but also dissolve and eliminate iron compounds of the tissues, while the danger of contamination by exogenous iron also arises. The tissues are saturated with water, and wet weight determinations are consequently unreliable. In determining the total iron of the tissues after this treatment, the measured iron includes not only the storage-iron fraction but also the chromoprotein iron of myoglobin and cell haemines. From the standpoint of the present investigation, it is important that *the placental structures and the foetus cannot be rendered free from blood in this way.*

2. The two tissue iron fractions may be isolated from each others and determined separately. Lintzel, 1930, 1933, and Schairer and Rechenberger, 1944, 1948, determined the total iron content of tissues homogenised in water. The haemoglobin in the suspension as such, or stabilised as carbon monoxide haemoglobin, was measured, and the haemoglobin-free tissue iron estimated by subtraction of the calculated haemoglobin iron value.

Haemoglobin may also be extracted from a homogeneous tissue suspension as hydrochloric acid haematin into a supernatant layer of ether-alcohol (Schaefer, 1940; Kooyman, 1949; Roth and coll., 1951). Determination of the total iron content of the residue after complete extraction gives a measure of the tissue iron free from haemoglobin iron. Scott, 1945, devised a method of determining "free iron of tissue extracts" including extraction by Schaefer's method of haematin with ether as hydrochloric acid haematin. The isoamyl alcohol extract finally arrived at was spectrophotometrically analysed for the "free tissue iron" as iron-thiocyanate. Crystalline rat haemoglobin was shown to give a reaction corresponding to 6 % free iron.

The extraction method used by Schaefer and others does not seem to be superior to methods using acid hydrolysis of the storage-iron complexes. The addition of n hydrochloric acid to the suspension and incubation at 37° to convert the haemoglobin to acid haematin seems to split off haemoglobin iron in

essentially the same way as does acid hydrolysis. Furthermore, the possibility of incomplete extraction of the acid haematin may introduce an uncertain error (Scott, 1945), and the method is in any case inconvenient.

3. As previously mentioned, the storage-iron fraction may be determined by acid hydrolysis of the iron-protein complexes. An account has been given of the theoretical basis for this method, as well as of the possible liberation of some haemoglobin iron under certain conditions.

It should be noted that it was not my purpose to isolate and determine a well defined storage-iron fraction in the absolute sense. As a matter of fact, this is not possible with the available methods and our present incomplete knowledge of the chemistry and physiology of different fractions of the body iron.

The purpose of this investigation was to determine the tissue iron content of certain placental structures and the foetus — this iron being hydrolysable under standardised conditions — in order to obtain commensurable values in different animals and groups of animals. The method I have used seems superior in these respects to alternative methods.

i. *Discussion of the method used*

It is of great importance for the accuracy of the method that the biological material is completely homogenised. The suspensions obtained were in fact macroscopically and microscopically homogeneous and did not leave a visible sediment after standing 24 hours. The good reproducibility of the method, as shown in the double determinations performed, is also evidence of the uniform distribution of the biological material in the suspension.

As regards the hydrolysis, it was essential to establish whether or not it liberated significant amounts of haemoglobin iron, which would make the measured values incommensurable in view of the different blood contents of the tissues examined. The following experiments were carried out to check this.

1 gm of whole blood was analysed. The mean of five analyses performed did not differ significantly from the blank values. Moreover, the analyses of the actual tissues did not differ from those obtained when haemoglobin was stabilised as carbon monoxide haemoglobin in the aqueous tissue suspensions acc. to Barkan and Berger, 1928. (Illuminating gas was bubbled through suspensions of the different tissues for some 10 minutes. To establish that most haemoglobin was transformed into carbon monoxide haemoglobin, diluted whole blood

Table V.

Effect of carbon monoxide upon the photometric extinction values.

Tissue	Photometer extinction readings	
	Untreated tissue suspension	After bubbling through illuminating gas
Foetus I	0.060 0.059	0.061 0.060
Foetus II	0.050 0.049	0.051 0.052
Placenta I	0.051 0.050	0.050 0.050
Placenta II	0.046 0.047	0.046 0.046
Liver I	0.230 0.228	0.232 0.230
Liver II	0.298 0.299	0.300 0.301

was also subjected to the same procedure. The absorption curve obtained was in full accordance with that of carbon monoxide haemoglobin.)

The results of these experiments are listed in Table V.

There thus seems to be little reason to suppose that iron liberated from haemoglobin affects the results obtained with the author's method.

Another factor that might possibly have affected the values obtained was the amount of the trichloracetic acid precipitate. It was found that when a large amount of the tissue was used in the analysis, with a corresponding increase of the precipitation product, some iron was retained in the residue upon filtering, to an extent depending on the amount of tissue used. The observation of this source of error was the reason for the constant, rather high, tissue dilution used in all the analyses. Another precaution was that the residue was always washed with small volumes of trichloracetic acid to ensure a maximum yield.

The rest of the procedure was in accordance with the principles of serum iron determination outlined by Heilmeyer and Plötner, 1937, and modified by Vahlquist, 1941, except that I preferred to complete the final solution to a definite volume.

2. Determinations of the total iron in tissues. (The Method 2.)

The determination of the total iron in tissues is possible only after complete destruction of the organic matter. This is accomplished by ashing, — wet or dry.

Dry ashing. This is now generally carried out in electric muffle ovens. The duration of the process is relatively long, and some sources of error might contribute to incorrect values if neglected. It has been pointed out that iron may be lost as ferric chloride if the temperature is raised too high. Furthermore, the product from dry ashing may contain iron compounds of rather low solubility.

Wet ashing. This method has none of the disadvantages of the dry ashing method, and is the method I adopted.

a. *Principle*

The principle of wet ashing and the subsequent determination of iron in the clear solution is well known, and will not be described here. (For references see Hemmeler, 1951)

b. *Apparatus*

The same as described under Method 1.

c. *Reagents*

1. *Concentrated sulphuric acid* pro analysi (Baker).
2. *Concentrated nitric acid* pro analysi (Baker).

3—8. *Paranitrophenol, ammonium hydroxide, sulphuric acid, hydroquinone, o-phenanthroline hydrochloride* and *iron standard solutions* as used in Method 1.

d. *Procedure*

The homogenising procedure has already been described under Method 1.

1. 5 ml of the homogeneous suspension were transferred to a Kjeldahl vessel. 1.5 ml conc. sulphuric acid and 1 ml conc. nitric acid were added and the vessel agitated for one min. The vessel was gently heated on an electric sand-bath until nitrous fumes ceased to be evolved. The liquid was then allowed to cool, whereupon another 0.5 ml of conc. nitric acid were added; this was repeated until a completely clear solution was obtained and the nitrous fumes

were completely expelled. Some bidistilled water was added, and heating continued. After cooling, the contents of the Kjeldahl vessel were transferred to a 25 ml volumetric flask, the Kjeldahl vessel being repeatedly rinsed with bidistilled water to ensure complete transference of the ashing product. The volume was made up to 25 ml.

2. 2 ml of the clear solution was pipetted off into a 5 ml volumetric flask and subjected to the treatment described under procedure 4—6 in Method 1.

e. *Calculation of the value*

The total amount of iron in the tissue, expressed as mg of iron per 100 gm of tissue wet weight, was arrived at according to the following formula:

$$\frac{E \times K \times 275}{20}$$

E denotes the extinction reading of the photometer, corrected against the blank value.

K denotes the constant obtained in the photometric measurements.

f. *Accuracy of the method*

This was checked by adding a known amount of iron to the material analysed (33.3 γ of iron — i.e. 5 ml of the 666 γ % standard iron solution, — were added to 5 ml of tissue suspension, the iron content of which is given as the mean value of 5 analyses performed). The results of these determinations are given in Table VI.

Table VI.

Determinations of total iron in biological material with added iron.

Amounts of iron in γ			
I		II	
Calculated	Measured	Calculated	Measured
56.51	55.00	62.13	60.63
56.51	54.06	62.13	60.94
56.51	55.31	62.13	61.88
56.51	57.50	62.13	61.56
56.51	55.94	62.13	61.88

Table VII.

The error of the measurement (σ_m) in determinations of the total iron concentration of foetal bodies.

Tissue examined	No. of double determinations	Mean in mg per 100 gm wet weight	σ_m	σ_m per cent of mean
Foetus	16	4.64	0.12	2.6

The reproducibility of the method has been computed statistically from a series of double determinations. The results are given in Table VII.

It will be seen that the methodic error, σ_m, constitutes 2.6 % of the mean value obtained.

3. Other methods employed

a. *Haemoglobin* was determined as alkaline haematin according to the method formulated by Clegg and King, 1942, and recommended by Delory, 1949. To convert haemoglobin into alkaline haematin, blood was dissolved in n/10 sodium hydroxide solution. For comparison, an alkaline haematin standard was prepared equivalent to a concentration of 14.8 gm haemoglobin per 100 ml of blood. This standard was made up by dissolving pure crystalline haematin in a Sörensen buffer of pH 9.4, and it was always kept in an ice-box when not in use. The determination was carried out with a DU Beckman quartz spectrophotometer, and the haemoglobin concentrations in gm per 100 ml of blood calculated according to the formula:

$$\frac{\text{Test reading}}{\text{Standard reading}} \times 14.8.$$

b. *The number of red blood cells* per cu.mm. of blood was determined by counting in a Buerker chamber, the same chamber being used throughout the investigation. 40 C-squares were counted for each determination.

c. *The haematocrite* determinations were performed with the haematocrite equipment of the Corda-centrifuge. The haematocrite tubes

were washed out with 5 % heparin solution and allowed to dry overnight before use. The centrifuge was run at 3 000 r.p.m. for 20 min. A further period of 10 min. did not change the measured value.

d. *Plasma iron determination.* This was performed in accordance with the method of Heilmeyer and Plötner as modified by Vahlquist, except that dilution to a fixed final volume was preferred. The haematological determinations were performed by the same assistant during most of the investigation. When another assistant was temporarily employed, her values were tested against those of the usual assistant before being entered in the results. Double determinations were made throughout.

4. Statistical methods used

On account of the small number of cases in each group, the material has been dealt with statistically by the methods worked out by Fisher, 1936 and Gosset. The significance of a difference has been estimated throughout by considering the ratio of the difference to the standard error, t, and the number of degrees of freedom. The significance is denoted by P, which signifies the probability that a difference at least as large as that observed could arise by random variation. The following significance levels have been used.

Where $P \leqq 0.003$	the difference is termed statistically significant (**)
Where $0.003 < P < 0.02$	the difference is termed statistically probable (*)

The first case ($P \leqq 0.003$) corresponds to = 3 times, and the second case to 2.3—3.0 times the standard deviation of the normal curve when the number of cases is very large.

In Table XII	$P<0.05$	stands for $0.02 < P < 0.05$
	$P<0.02$	stands for $0.01 < P < 0.02$
	$P<0.01$	stands for $0.003 < P < 0.01$
	$P<0.003$	stands for $0.001 < P < 0.003$

CHAPTER VII

The test animals and investigation technique

Some 300 white unmated female rats were used in this part of the investigation. The material included consists of 132 animals (see Table VIII). All the animals were guaranteed to be of the same strain and reared by the same breeder. The average age was 3 months when delivered to the Institute. They were kept there some 7 days before mating, and animals showing signs of physical disorder were eliminated. During the experimental period they were kept on the same balanced diet, supplemented with vitamin E in the form of 'Evimin' (Astra) in drops upon the bread. To begin with, all the females were kept away from the males, and their sexual cycles were followed by histological examination of the vaginal smears as described by Long and Evans, 1922. When on heat, the females were brought together with the males overnight, and possible conception was determined in the morning by the appearance of the vaginal plug. Pregnancy was calculated from the day this plug was found. The female rats were then weighed, and this weight was taken as the initial experimental weight (see Table VIII).

The test animals were divided into two main groups as follows:

1. '*Normal pregnancy group.*' These rats were untreated during the experimental period of their pregnancy.

2. '*Anaemic pregnancy group.*' These rats were rendered haemorrhagically anaemic by daily bleeding. In all other respects they were treated like the animals of the foregoing group.

The tow main groups were subdivided into 5 subgroups, depending upon the stage of pregnancy. Thus pregnancy was allowed to continue for 15, 17, 19, 20 and 21 days, each of these periods corresponding to a subgroup (see Table VIII). The evening before killing, the rats were deprived of all solid food and only allowed to drink water ad lib.

A. Experimental technique

The initial haematological values (haemoglobin concentration, number of red blood cells and haematocrite values) of the anaemic pregnancy group were obtained from the tail blood on the day preg-

nancy was established, and, together with the weights, were recorded as the initial experimental data of this group.

The values for tail blood might admittedly not be representative, as compared with the values for the heart blood used in the rest of the investigation. However, puncturing the heart was shown very often to cause injuries not detectable until autopsy. Furthermore, the reason for establishing the haematological initial values was to ascertain that the animal material was uniform in this respect.

B. Technique used to establish a standardised experimental anaemia

The animals belonging to the anaemic pregnancy group were bled daily from the first day of pregnancy. The method of bleeding followed the technique used by SUNDELIN, 1945. The rats were put under light ether anaesthesia, wrapped in a towel, the tail drawn out, cut with a pair of scissors and dipped into a graduated glass partially filled with hot water. The water level was noted when the tail had been introduced into the vessel. The usually very rapid bleeding was allowed to continue until about 2 ml of blood were lost. The bleeding ordinarily ceased when the tail was removed from the water, but if it did not, it was stopped with a thin film of celloidin. This procedure rapidly brings the animal to an anaemic condition, as was shown by SUNDELIN. With the method employed, the experimental anaemia of haemorrhagic, type observed on the 15th day of gestation remained constant throughout the rest of the observed gestation, as is shown by the haematological characteristics, values of plasma iron and storage-iron of the liver reproduced in Table VIII.

This table contains the experimental results for all the characteristics of the pregnant test animal.

C. Further experimental procedures

The animals to be used in experiments on a particular day were attended to in the morning. Each animal was weighed and the weight noted. Under light ether anaesthesia the abdomen was opened, and the heart exposed through an incision in the diaphragm. The right atrium was punctured by a platinum needle fitted to a whole glass syringe, which was completely dry. The blood was allowed to fill

the syringe by the force of the heart's muscular work, and was not sucked up. In this way some 5 ml of blood were obtained, in most cases completely free from visible haemolysis. The blood was transferred to a centrifuge tube, from which blood specimens for haemoglobin, number of red cells and haematocrite determinations were pipetted off. The rest was centrifuged at 3 000 r.p.m. for 10 min., and the plasma portion set aside for plasma iron determination. A piece of the median lobe of the liver was cut out and placed in a closed weighing vessel, weighed in advance.

The two uterine branches were dissected free and cut out, together with the vagina. The organ was placed on a clean glass plate and the uterine wall incised above each egg swelling. Incisions were made into the amniotic cavity, whereupon the foetus was spontaneously born through the opening, together with amniotic fluid. The umbilical cord was burnt off with a red-hot glass rod close to the umbilicus, to prevent bleeding. The visceral yolk sac wall was freed, which is easily done, and cut away from the umbilical cord. The specimen thus obtained was a circular membrane, with a hole in its centre where the umbilical cord had been attached (see Plate I). The discifórm allantoic placenta was then removed from its uterine attachment. As the separate structures of the eggs were obtained, they were all immediately placed into previously weighed closed weighing vessels, one for each kind of material, to prevent loss of water. After weighing the weights were noted, as well as the number of eggs in each case. — Only animals pregnant with 8—12 foetuses were recorded —. The tissues were then homogenised, as described, and the amounts of hydrolysable iron in the foetal, yolk sac and placental tissues determined. The foetal tissues were also subjected to total iron determinations.

D. Disscussion

In this investigation, anaemia is taken to be a condition in which the animal organism, continually deprived of whole blood, is subject to a marked dimunition in the amount of haemoglobin circulating in the red cells. Considerable amounts of iron are lost with the tapped haemoglobin and serum. The rat's blood volume is rather small, the reported values ranging between 4 and 8 ml per 100 gm of body weight, according to the method used. Berlin, Huff, Van Dyke, and Hennessy, 1949, using labelled iron and phosphorous, determined it to

be 4.59 ml per 100 gm of body weight. As we know that the rat's blood contains some 0.5 mg of iron per ml (Scott and McCoy, 1944), it follows that the bleedings performed mean the loss of a large proportion of the total blood and iron. Furthermore, as the animals were pregnant and as, according to Widdowson and McCance, 1948, the rat mother has to provide about 2.8 mg of iron for her full term litter, it is evident that an iron deficiency soon develops unless the loss of iron is compensated for by absorption and mobilisation of available iron depots. Laurell, 1947, investigated the effect of chronic loss of blood by blood donors, and found that the transferrin concentration increased at the same time as the serum iron level fell, these being the conditions for increased iron absorption and iron mobilisation from the depots. However, Hahn, 1937, concluded from a series of experiments on iron-depleted dogs that the storage iron is insufficient to compensate for more than 30—35 % of the circulating haemoglobin. Finch and coll., 1948—1952, stated that the iron stores of the body must be depleted before iron deficiency is established in the circulating red cell mass. They found repeated bleedings of human subjects to deplete the iron stores, which were rebuilt but slowly.

The bleeding method used in this investigation was found to deplete the iron stores of the liver to a low, constant level (see Table VIII). The circulating haemoglobin was shown to decrease appreciably, as did the number of the red cells and the plasma iron concentration, all the values being at the same, low level throughout the experimental period.

The pregnant rat subjected to such haemorrhagic anaemia is thus unable to compensate for its lost iron by increased absorption or mobilisation of iron from the depots. Moreover, the degree of this experimental anaemia seemed to be well established and constant from the first day of examination i.e. the 15th day of gestation, and persisted unchanged during the rest of pregnancy examined (see Table VIII).

The considerable loss of blood involves the depletion of other blood constituents than haemoglobin, e.g. serum proteins and electrolytes. It may be asked whether these factors lead to any distortion of the experimental results. The animals appeared quite normal, however, were without signs of lassitude or physical disorder, but gained in weight less than those of the normal pregnancy group (see Table VIII).

Sundelin, 1945, applied a similar method to non-pregnant test

Table VIII.

Weight, haematological characteristics, plasma and hydrolysable liver iron values of the pregnant rats.

Day of pregnancy	No. of ind.	Weight in gm		No. of red blood cells mill./cu.mm	Haemoglobin gm/100 ml	Haematocrite %	Plasma iron, γ %	Hydrolysable iron of the liver mg/100 gm fresh tissue
		at conception	during the experiments					
				Normal pregnancy group				
15	15	162 ± 5.0	204 ± 5.9	7.5 ± 0.2	13.3 ± 0.2	41.3 ± 0.9	157.1 ± 7.1 [1]	12.4 ± 0.6
17	18	174 ± 5.9	220 ± 6.4	7.4 ± 0.2	13.2 ± 0.2	41.2 ± 0.8	151.9 ± 5.0	10.9 ± 0.4
19	17	161 ± 6.2	226 ± 7.9	7.4 ± 0.2 [2]	13.4 ± 0.2	40.1 ± 1.0	150.1 ± 6.8	10.2 ± 0.6
20	15	173 ± 5.5	243 ± 8.4	6.9 ± 0.2	12.5 ± 0.3	38.1 ± 0.9	133.0 ± 12.6 [3]	8.1 ± 0.5
21	18	167 ± 3.2	254 ± 6.7	6.9 ± 0.2	12.3 ± 0.2	38.0 ± 0.6	131.2 ± 5.7	7.4 ± 0.4
				Anaemic pregnancy group				
15	10	149 ± 6.0	173 ± 7.0	3.4 ± 0.2	8.0 ± 0.4	28.1 ± 1.4	68.1 ± 6.4	3.7 ± 0.4
17	9	150 ± 3.3	180 ± 6.0	3.4 ± 0.2	7.9 ± 0.5	22.9 ± 1.2	80.6 ± 9.4	3.9 ± 0.3
19	10	161 ± 5.8	204 ± 8.1	3.2 ± 0.2	6.9 ± 0.4	22.2 ± 1.4	74.8 ± 9.2 [4]	3.1 ± 0.3
20	10	164 ± 5.8	211 ± 6.7	3.2 ± 0.2	7.5 ± 0.5	25.4 ± 2.1	61.6 ± 4.6 [5]	3.5 ± 0.4
21	10	159 ± 4.4	207 ± 7.8 [5]	3.1 ± 0.5	7.2 ± 0.7	25.0 ± 1.7	70.9 ± 4.8	4.0 ± 0.4

[1] Mean of 14 ind. [2] Mean of 16 ind. [3] Mean of 13 ind.
[4] Mean of 8 ind. [5] Mean of 9 ind.

animals of the same type, and found no decrease of the serum proteins. The alternative of rendering the animals anaemic by a diet devoid of iron is not preferable. To obtain a commensurable degree of iron deficiency dietetically would require a considerable time, even prior to conception, and conception would be more difficult to obtain, acc. to Sundelin. Intrauterine deaths of one or several foetuses in several animals of the anaemic pregnancy group were observed — these animals were of course eliminated — and as this was never observed to happen in the normal pregnancy group, it was interpreted as a consequence of the experimental anaemia. The haemorrhagic anaemia arrived at with the methods employed seems to establish the most suitable form of experimental anaemia for the purposes of this investigation.

E. Comments on the characteristics of the test animals

All the characteristics for the test animals, i.e. the maternal rats are presented in Table VIII.

The methods used were described in detail in a foregoing chapter.

It will be seen that the weight distribution in the two main groups and in the different subgroups as far as the weight at conception is concerned is fairly uniform. The animals of the anaemic pregnancy group were shown to gain less in weight, the weights on the day of operation being appreciably less than the corresponding values for the normal rats. (This was the case also with the foetuses of anaemic pregnancy.) Table VIII further indicates that in the normal pregnancy group both the red cell count and the haemoglobin concentration tended to fall towards end of pregnancy. The plasma iron values decreased in a statistically significant manner on the 20th day of pregnancy and remained at this low level on the 21st day. Regarding the amount of iron, determined as hydrolysable iron of the tissues (defined in a foregoing chapter) and corresponding to the storage-iron, it is seen that its amount per unit weight of the liver also decreased in a statistically significant manner on the 20th day of gestation and remained low on the 21st.

In the anaemic pregnancy group, the mean values of red cell counts, the haemoglobin and plasma iron concentrations, and the amount of hydrolysable iron per unit weight of liver tissue remained unchanged throughout the observed period of gestation, indicating a constant iron deficiency in the maternal animals.

The haematocrite determinations reveal that there was in the anaemic pregnancy group a fairly constant hydraemia as compared with the normal pregnancy group.

F. Discussion of the characteristics obtained for the test animals

For the last four or five days of pregnancy VAN DONK, FELDMAN, and STEENBOCK, 1934, found the physiological anaemia of rat pregnancy to be most pronounced, with haemoglobin values averaging 11 to 13 gm per 100 ml of blood and red cell counts of 6 to 7 millions per cu.mm. of blood. In the present material, representing normal pregnancy from the 15th to the 21st day of gestation, the values, though obtained from heart blood, are somewhat higher and a tendency to decrease towards term was not noted until the 20th day of pregnancy.

The normal haematocrite value for the rat is considered to be 45—50 %. In the present material of normal pregnancy, it averaged 40 %.

As far as I know, no plasma iron determinations have been carried out in pregnant rats. The values of plasma iron concentration in the normal non-pregnant rat seem to be rather high, 200—250 γ % (Scott and McCoy, 1944, and Kinney, Hegstad, and Finch, 1949). Using the method of Heilmeyer and Plötner, I obtained for the normally pregnant animals values averaging 150 γ %, and moreover, a statistically significant decrease appearing on the 20th day of gestation. This might be compared with the slight decrease in the serum iron concentration observed towards term in human pregnancy (Sundelin, 1942 and others). Regarding the amounts of storage-iron per unit weight of the liver, it has been pointed out that it decreases during pregnancy (Smythe and Miller, 1929; Steenbock, Semb and van Donk, 1936, and Widdowson and McCance, 1948). The latter authors found the liver of non-pregnant female rats to contain 34 mg of inorganic iron per 100 gm of wet tissue on the 200th day of age, whereas at the end of pregnancy it had fallen to little more than 8 mg / 100 gm, a figure corresponding well to that found by myself on the 20th and 21st days of normal pregnancy in rats. The corresponding characteristics of the anaemic pregnancy group bear out the assumption of an iron deficiency, representing a depletion of the iron depots of the mother and a considerable decrease in her circulating blood iron, sufficient to render her incapable of delivering iron to the conceptus other than that continuously absorbed from the intestine.

CHAPTER VIII

The Results

The following abbreviations are used:

Total iron = Total Fe
Hydrolysable iron = Hy Fe
Allantoic placenta = All. placenta
Visceral yolk sac membrane = Y.S. placenta

The two placental organs of the rat taken together are called the total placental barrier.

Table IX.

Mean content of total Fe in the foetus and the Hy Fe of the foetus and its placental structures at different stages of gestation in normal and anaemic animals.

Day of pregnancy	No. of ind.	Total Fe, γ	Hy Fe, γ			
		Foetus	Foetus	Total placental barrier	All. placenta	Y.S. placenta
			Normal pregnancy group			
15	15	3.9±0.5	1.9±0.2	3.0±0.1	2.0±0.2	0.9±0.1
17	18	17.5±1.7	8.4±0.8	7.3±0.5	5.1±0.3	2.2±0.2
19	17	70.3±3.8	31.8±1.8	12.9±0.4	8.6±0.4	4.3±0.2
20	15	123.4±6.4	62.0±2.9	13.3±0.7	8.6±0.4	4.8±0.4
21	18	207.3±8.3	93.6±5.3	14.8±0.8	8.9±0.4	5.9±0.5
			Anaemic pregnancy group			
15	10	2.7±0.3	1.4±0.1	3.1±0.1	2.6±0.1	0.5±0.04
17	9	10.7±1.2	5.6±0.6	5.8±0.4	4.5±0.4	1.3±0.1
19	10	38.1±4.0	17.8±1.8	8.2±0.5	6.3±0.5	1.9±0.2
20	10	78.4±5.0	32.7±3.2	9.5±1.0	6.8±0.9	2.8±0.3
21	10	95.2±8.1 [1]	30.1±3,7	9.5±1.0	6.4±0.8	3.2±0.3

[1] Mean of 9 ind.

A. The total and Hy Fe in the individual foetal organism and the Hy Fe in its placental organs at different stages of gestation

1. Normal pregnancy group

a. *Total and Hy Fe of the individual foetus*

The amounts of total and Hy Fe per foetus are given graphically in Fig. 7 a and numerically in Table IX.

It will be seen that the amounts of *total Fe* increase rapidly towards term (Table IX). Some two-thirds of the total Fe found on the last day of gestation is delivered in the course of the 19th and 20th days of pregnancy, and approximately two-fifths during the 20th day.

It is also clear that the *Hy Fe* fraction obtained under the given experimental conditions constitutes about half the total Fe fraction of the foetus throughout the observed stages of gestation, and thus increases towards term in the same way as was noted for the total Fe content of the foetus (see Fig. 7 and Table IX).

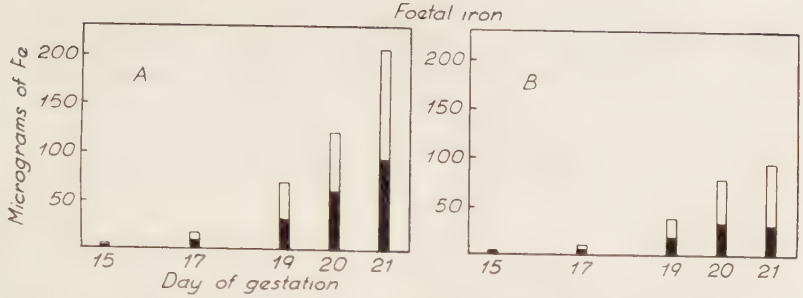


Fig. 7. Total Fe content of the foetus during the observed period in normal (A) and anaemic (B) pregnancies. Shaded parts represent the Hy Fe fraction.

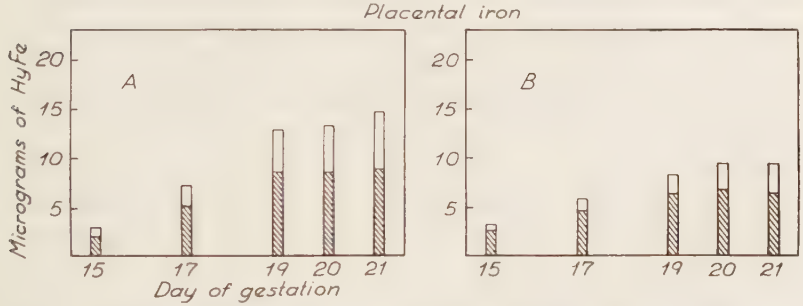


Fig. 8. Placental content of Hy Fe during the observed period in normal (A) and anaemic (B) pregnancies. Hatched parts represent the allantoic placenta and unshaded parts the yolk sac one.

b. *Hy Fe in the placental organs of the foetus*

The content of Hy Fe in the placental organs of the individual egg is indicated in Fig. 8 A and Table IX. It should be noted that the mean values of Hy Fe in the two placental organs, taken together as the *total placental barrier*, increase continuously during gestation until the 19th day, the values on the 15th day being only about a quarter of the value on the 19th day. From this day on, however, *no significant increase takes place* (the difference between the 19th day value — 12.9 — and that of the 21st day — 14.8 — being only 1.9 ± 0.9).

The *all. placenta* seems to hold about two-thirds of the Hy Fe contained in the total placental barrier of the egg, in all observed stages of gestation (see Table IX and Fig. 9 B). The *Y.S. placenta* therefore accounts for only one-third. This constant relation between

the two placental organs is apparent from the fact that individually *they show the same variation of Hy Fe content as the total placental barrier*, see Fig. 8 A.

2. Anaemic pregnancy group

a. *Total and Hy Fe of the individual foetus*

The values of the total and Hy Fe are apparent from Table IX and the diagram given in Fig. 7 B.

There is a *continuous increase* in the *total* Fe content of the foetal organism from the 15th day of gestation, including the 20th day. *The increase noted between the last two days of gestation is slight*, however, and as a matter of fact, not statistically significant. It may be seen that only about half the total Fe contained in the foetus on the last day of gestation is delivered during the 19th and 20th days of pregnancy. Only an insignificant amount of iron is transferred to the foetus between the 20th and 21st days.

As regards the *Hy Fe* fraction, this increases from day to day, although *the increase does not extend to the 21st day* of gestation, for which the value is about the same as that for the 20th. The Hy Fe of the foetal organism is approximately half of the total Fe, even on the 20th day of gestation. The ratio 1/3 on the 21st day is explained by the above-mentioned insignificant increase in the total Fe and the stationary value of the foetal Hy Fe.

Comparing the two iron fractions of the *anaemic group* with those of the *normal pregnancy group* (see Fig. 7 A and B and Table IX), it is obvious that the *total Fe of the individual foetus is at all observed stages of gestation far below the corresponding value for normal pregnancy. This becomes more evident as gestation proceeds, and is most pronounced on the 21st day of pregnancy.* The increase of the *total Fe* fraction of the foetus with the duration of pregnancy is thus *significantly less* in this group than in the normal pregnancy group, principally due to the insignificant increase in total Fe between the last two days of pregnancy. The corresponding increase in the normal pregnancy group in this interval is about five times as high.

Regarding the *Hy Fe* of the foetal body, it is increased in much the same way as the total Fe, the increase with the duration of gestation being *less pronounced in the anaemic group* than in the normal pregnancy group. There is no increase at all in this fraction between

the last two days of gestation in the anaemic pregnancy group, whereas for the corresponding period in the normal group an appreciable increase occurs. *Thus, the increase in both the total and the Hy Fe with advancing gestation is significantly higher in the normal pregnancy group than in the anaemic, the difference becoming more pronounced towards term.*

b. *Hy Fe in the placental organs of the foetus*

The results of these determinations are presented in Table IX and Fig. 8 B.

The mean amounts of Hy Fe in the *total placental barrier* rise *continuously with duration of pregnancy up to the 19th day, after which no further increase is statistically demonstrable.* (The difference between the 19th day value, — 8.2 — and that of the 21st day, — 9.5 — is 1.3 ± 1.1, which is not statistically significant.) If the two placental organs are considered separately, we see that in the *all. placenta*, which contains most Hy Fe, *the Hy Fe increases significantly up to the 19th day of pregnancy*, after which only an insignificant increase occurs during the rest of the observed period of gestation.

The same holds true for the *Y.S. placenta*, although *the Hy Fe content of this placental organ increases proportionally more than that of the all. placenta.* On the 15th day of gestation it contains only about one-sixth of the Hy Fe of the total placental barrier, but on the 21st day the proportion has increased to about one-third (see Table IX and Fig. 9 A). This increase in the yolk sac part of the placental iron is statistically significant ($P < 0.001$).

Comparing these values with those obtained from the *normal pregnancies*, it will be seen that the mean amounts of Hy Fe in the *total placental barrier from the 17th day of gestation on are lower than those of the normal pregnancy group.* It increases in the same way up to the 19th day, *though the increase is significantly less pronounced* than that of the normal group. On the 15th day of gestation the Hy Fe content of the total placental barrier equals that of the normal group.

The Hy Fe content of the *all. placenta* reveals the same tendency as that of the total placental barrier, the values being significantly lower than those of the normal pregnancy group from the 17th day on, and the increase with duration of pregnancy up to the

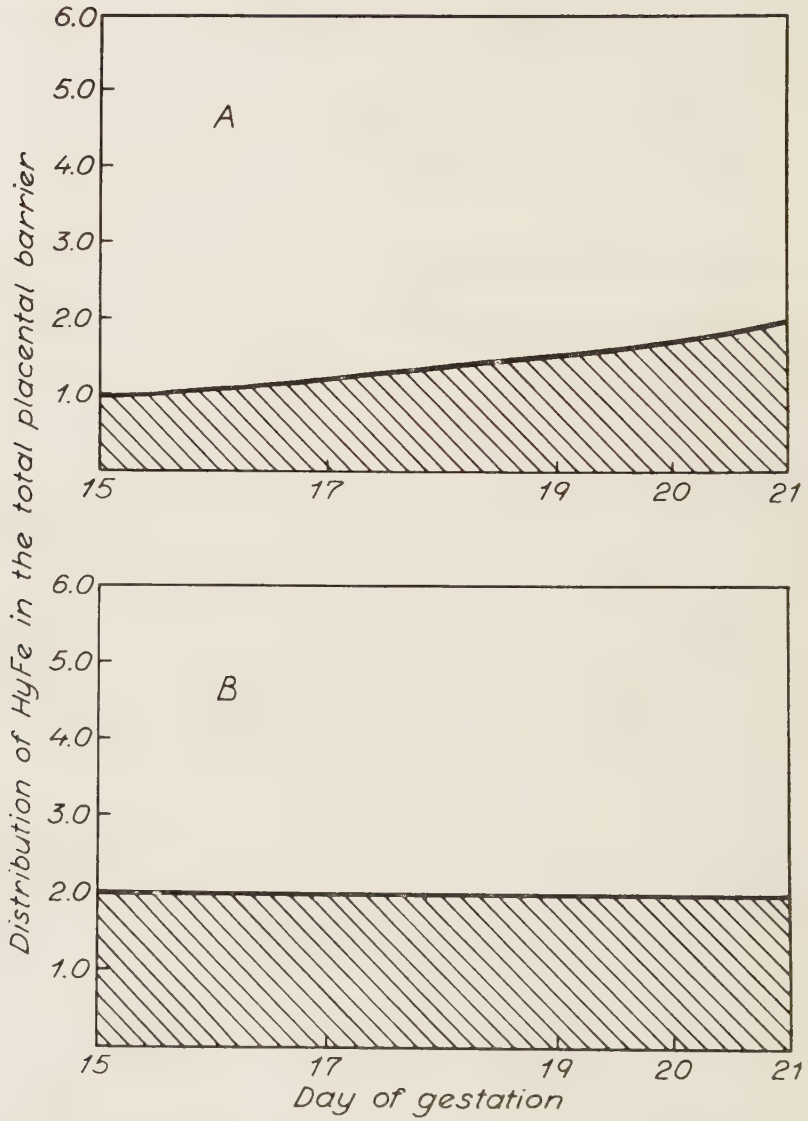


Fig. 9. The distribution of Hy Fe in the total placental barrier during the observed period in anaemic (A) and normal (B) pregnancies. Yolk sac fraction is represented by the hatched area.

19th day is significantly less pronounced. It should be noted that the Hy Fe content of the all. placenta on the 15th day of gestation in the anaemic animals is higher than the corresponding value for the

normal ones, making the Hy Fe content of the total placental barrier in the anaemic cases equal to that of the normal material.

Throughout the observed period of gestation, the Hy Fe content of the *Y.S. placenta* is significantly lower than that of the normal pregnancy group, and the increase with advancing gestation is significantly less pronounced as well. In the normal pregnancy group the ratio between the amounts of Hy Fe in the all. and Y.S. placentas remained relatively constant, the latter constituting approximately one-third of the Hy Fe contained in the total placental barrier. In the anaemic pregnancies, however, the above ratio is only 1/6 on the 15th day, after which it rises to 1/3 on the 20th day of gestation — the same value as for the normal group (see Fig. 9). The difference in this respect between anaemic and normal pregnancies is statistically significant.

To sum up, the Hy Fe content of the all. placenta as well as that of the total placental barrier, is from the 19th day of gestation significantly lower than the corresponding values for the normal pregnancy group; the same is true for the Y.S. placenta from the 15th day on. The increase in Hy Fe with duration of pregnancy up to the 19th day of gestation is common to both the placental organs and to both the anaemic and normal groups though significantly more pronounced in the normal pregnancy group. In the anaemic pregnancies, the Y.S. accumulates proportionally more Hy Fe with advancing gestation than does the all. placenta, — unlike the constant ratio between the Hy Fe content of the two placental organs for all observed stages of gestation in the normal pregnancy group.

B. 'Placental iron turnover'

It might be of interest to calculate the approximate turnover rate of Hy Fe in the placental structures, taken together as the total placental barrier. If this iron is supposed to be involved in the transfer of iron from mother to foetus, and if *the whole amount determined by analysis* in a certain space of time is delivered to the foetal organism, assuming no iron is transferred in the opposite direction, a measure of the minimum turnover rate of the placental iron might be arrived at.

We note that in the *normal pregnancies* the amount of iron accumulated in the foetus during the period from the 19th to the 20th day

Table X.

The placental iron turnover rate calculated for the last two days of gestation in normal and anaemic pregnancies.

Amounts of iron in γ	Normal cases	Turnover quotient	Anaemic cases	Turnover quotient
Total iron accumulated in the foetus from the 19th to the 20th day of gestation	53.1		40.3	
Mean of Hy Fe in the total placental barrier for the same period of gestation	13.7	3.9 ± 0.5	9.1	4.4 ± 0.7
Total iron accumulated in the foetus from the 20th to the 21st day of gestation	83.9		16.8	
Mean of Hy Fe in the total placental barrier for the same period of gestation	13.7	6.1 ± 0.8	9.1	1.8 ± 1.0

of pregnancy is 53.1 γ, and the mean value of the Hy Fe content in the total placental barrier during the 19th—21st day is 13.7 γ, it will be seen that the placental iron has to be exchanged some 4 times to furnish the foetal gain in iron observed. The corresponding value for the *anaemic cases* is found to be approximately the same (see Table X). Hence in this period of gestation, there seems to be no difference between normal and anaemic pregnancy.

When the period of gestation between the 20th and 21st days is considered, the value calculated for the *normal cases* is about 6, i.e. the placental iron has to be exchanged 6 times to give the amount of iron accumulated in the foetus during this time. In the *anaemic cases*, however, the corresponding quotient is only about 2. The difference between the quotient $83.9/13.7 = 6.1$ for the normal pregnancies and $16.8/9.1 = 1.8$ for the anaemic is 4.3 ± 1.3, and is statistically significant (see Table X).

Thus, under the conditions given earlier, the placental iron turnover rate during the period between the 19th and the 20th days of gestation is approximately the same in normal and anaemic cases. During the following equal interval between the 20th and 21st days of gestation, it is significantly slowed down in the anaemic cases as compared with the normal ones. In the normal pregnancies the placental iron turnover is maintained at approximately the same rate (see Table X).

Table XI.

Mean concentration of total Fe in the foetus and of Hy Fe of the foetus and its placental structures, at different stages of gestation in normal and anaemic animals.

Day of pregnancy	No. of ind.	Total Fe mg/100 gm	Hy Fe mg/100 gm			
		Foetus	Foetus	Total placental barrier	All. placenta	Y.S. placenta
			Normal pregnancy group			
15	15	2.39 ± 0.22	1.17 ± 0.07	1.58 ± 0.08	1.33 ± 0.07	2.34 ± 0.14
17	18	3.61 ± 0.23	1.75 ± 0.08	2.15 ± 0.10	1.88 ± 0.10	3.18 ± 0.22
19	17	5.10 ± 0.23	2.25 ± 0.10	2.63 ± 0.06	2.36 ± 0.09	3.33 ± 0.17
20	15	5.54 ± 0.24	2.79 ± 0.08	2.28 ± 0.09	2.06 ± 0.08	2.82 ± 0.20
21	18	5.62 ± 0.16	2.53 ± 0.11	2.14 ± 0.09	1.94 ± 0.09	2.52 ± 0.17
			Anaemic pregnancy group			
15	10	1.73 ± 0.11	0.92 ± 0.04	1.66 ± 0.05	1.67 ± 0.05	1.60 ± 0.12
17	9	2.21 ± 0.16	1.15 ± 0.06	1.87 ± 0.13	1.78 ± 0.12	1.98 ± 0.17
19	10	2.97 ± 0.21	1.39 ± 0.09	1.67 ± 0.09	1.71 ± 0.09	1.58 ± 0.20
20	10	3.83 ± 0.29	1.58 ± 0.12	1.67 ± 0.15	1.62 ± 0.20	1.85 ± 0.23
21	10	3.58 ± 0.31 [1]	1.14 ± 0.12	1.49 ± 0.14	1.40 ± 0.16	1.66 ± 0.13

[1] Mean of 9 ind.

C. Total and Hy Fe per unit weight of the foetal bodies and Hy Fe per unit weight of the placental organs at different stages of pregnancy

1. Normal pregnancy group

a. ***Total and Hy Fe per unit weight of the foetal bodies***

The values of *total Fe successively increase with the duration of gestation*, reaching the value of 5.5 mg per 100 gm of foetal body weight on the 20th day of pregnancy (see Table XI and Fig. 10 A). *From this day on no significant increase was noted.*

The corresponding values of *Hy Fe* are approximately half of the total Fe concentration throughout the observed period of gestation and thus follow the same course of development, increasing significantly ($P < 0.001$) up to the 20th day, when the value is 2.8 mg of Hy Fe per 100 gm of foetal body weight. See Fig. 10 A. On the 21st day the value is 2.5, but the decrease of 0.3 is statistically insignificant ($P > 0.5$).

Recalling the values of the content of total and Hy Fe for the individual foetus given earlier (see Table IX), it will be seen that the considerable increases in both the total and the Hy Fe during the last days of pregnancy coincides with concentrations of these two iron fractions in mg/unit of foetal weight which remain approximately unchanged on the last two days of gestation. The foetal weight increases considerably during this period.

b. ***Hy Fe per unit weight ot the placental organs***

The concentration of Hy Fe in the *total placental barrier* increases steadily and in a statistically significant manner from the 15th day of gestation (1.6) to the 19th (2.6) ($P < 0.001$), see Table XI and Fig. 10 A. *The values decrease on the 20th and 21st days, however* (2.3 and 2.1 respectively). The difference between the value on the 19th day and on the 21st day is 0.5, which is statistically significant ($P < 0.003$).

The all. placenta, which has the lowest concentration of Hy Fe of the two placental organs, shows the same tendency in its Hy Fe concentration as does the total placental barrier, i.e. the concentration values *increase up to the 19th day of gestation and from then on decrease*. See Fig. 10 B. The decrease noted between the 19th (2.36) and 21st days (1.94) is 0.42, and is statistically significant ($P < 0.003$).

The same applies to the *Y.S. placenta*, where the content of Hy Fe per unit weight of the organ increases with advancing gestation, reaching its highest value on the 19th day (3.3), after which a statistically significant decrease sets in during the rest of the observed period of gestation, the value on the 21st day being 2.5 ($P < 0.003$).

Thus, both the placental organs, individually and taken together as the total placental barrier, show a continuous increase in the Hy Fe content per unit weight up to the 19th day of pregnancy, after which a decrease occurs during the last two days of the observed period of gestation, see Fig. 13.

Comparing these findings with those regarding the Hy Fe content of the individual organs (see Table IX), it will be seen that the significant decrease in Hy Fe concentrations noted on the last days of gestation is a consequence of the placental organs' increase in weight, as the absolute iron content of the different placental organs remains approximately the same during this stage of pregnancy.

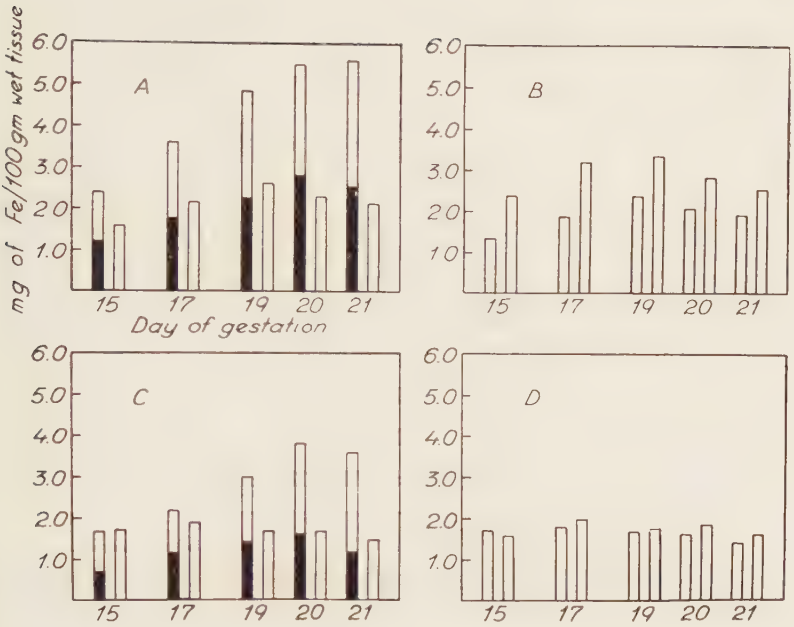


Fig. 10. Amounts of iron per unit fresh weight of the foetus and its placental organs during the observed period in normal (A and B) and anaemic (C and D) pregnancies. A and C: The first column represents the total Fe of the foetus (Hy Fe fraction shaded). The second, unshaded column represents the Hy Fe content of the total placental barrier. B and D: The first column represents the allantoic placenta Hy Fe, and the second the yolk sac Hy Fe.

2. Anaemic pregnancy group

a. *Total and Hy Fe per unit weight of the foetal bodies*

The *total Fe* concentrations throughout gestation vary in much the same way as in the normal pregnancy group. There is a statistically significant increase up to the 20th day of gestation, but from this day on no further increase occurs. The total iron concentrations are everywhere significantly lower than those of the normal pregnancies (Table XII and Fig. 10 and 11).

The amount of *Hy Fe* per unit of foetal also increases with advancing gestation up to the 20th day. The decrease occurring between the last two days of pregnancy is 0.44 ± 0.17 and is statistically probable. As was the case with the concentration of the total Fe, the concentration values are significantly lower than those of the normal pregnancy group (Table XII). In the anaemic cases there is a probable

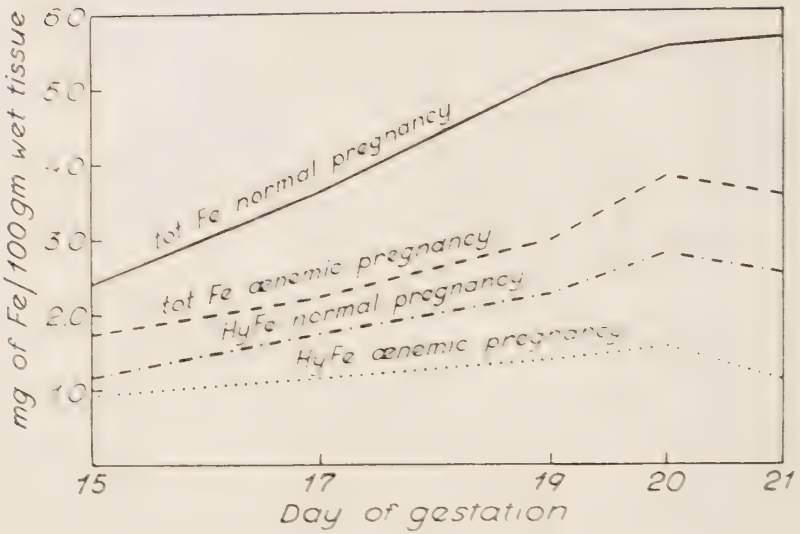


Fig. 11. Total Fe and Hy Fe per unit fresh foetal weight in normal and anaemic pregnancies.

decrease in Hy Fe concentration between the last two days of observed gestation, for which there is no equivalent in the normal pregnancies. (This is clearly demonstrated in Fig. 11.)

A comparison between the figures for the total and Hy Fe content of the individual foetal bodies (see Table IX) and the concentration values of the same iron fractions reveals that the foetal gain in weight during the period between the 20th and 21st day of gestation is small compared with that of the normal pregnancy group.

b. *Hy Fe per unit weight of the placental organs*

The two placental organs taken together as the *total placental barrier* exhibit a Hy Fe concentration which does not seem to vary in a definite way. The mean values for the different days might be random deviations from the same value. Variance analysis gives $0.02 < P < 0.05$. The value for the 17th day is 1.87 and for the 21st day 1.49. The difference, 0.38 ± 0.19, is not statistically significant.

Comparing these values with those for the *normal group*, it will be seen that in both groups the values for the 15th day are approximately the same. The subsequent increase in the normal pregnancies means

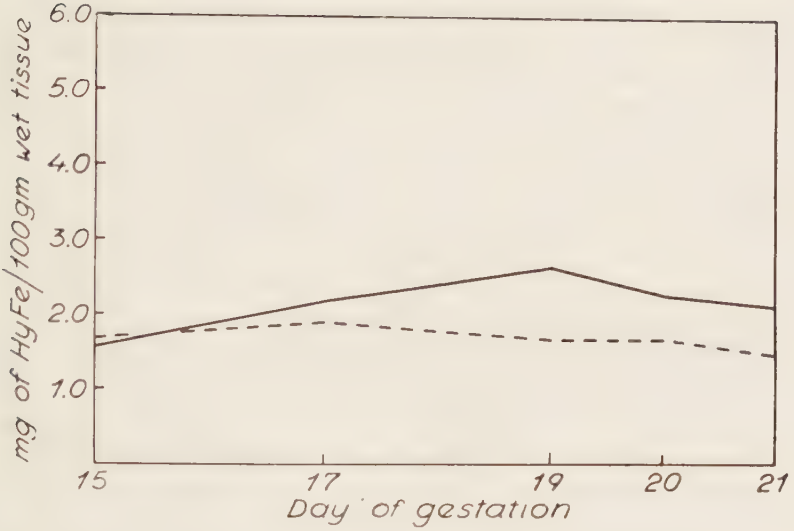


Fig. 12. Hy Fe content per unit fresh weight of the total placental barrier during the observed period in normal and anaemic pregnancies.
— — = normal pregnancy. — — — — = anaemic pregnancy.

Table XII.

Comparison of the concentration of Hy Fe in the foetus and its placental structures of the normal and anaemic groups. (The values on which the differences are based are given in Table XI.)

Day of pregnancy	Degrees of freedom [1]	Difference	P [2]	Difference	P [2]
		Foetus		Total placental barrier	
15	23	0.25 ± 0.093	<0.02	-0.08 ± 0.110	>0.05
17	25	0.60 ± 0.126	<0.001	0.28 ± 0.166	>0.05
19	25	0.86 ± 0.149	<0.001	0.96 ± 0.107	<0.001
20	23	1.21 ± 0.144	<0.001	0.61 ± 0.162	<0.001
21	26	1.39 ± 0.172	<0.001	0.65 ± 0.163	<0.001
		All. placenta		Y.S. placenta	
15	23	-0.34 ± 0.090	<0.001	0.74 ± 0.197	<0.003
17	25	0.10 ± 0.162	>0.05	1.20 ± 0.334	<0.003
19	25	0.65 ± 0.141	<0.001	1.75 ± 0.249	<0.001
20	23	0.44 ± 0.192	<0.02	0.97 ± 0.309	<0.01
21	26	0.54 ± 0.172	<0.01	0.86 ± 0.245	<0.003

[1] Degrees of freedom = the number of normal and anaemic ind. minus 2.
[2] P signifies the probability that the difference is due to random variation.

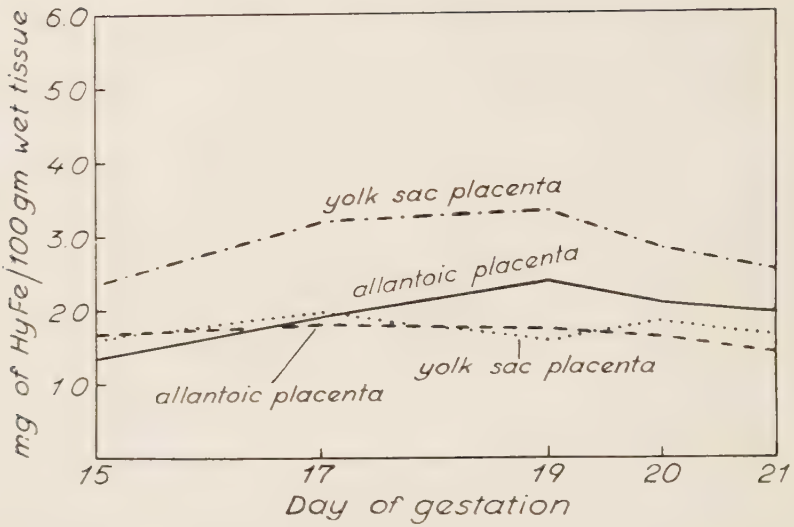


Fig. 13. Hy Fe content per unit fresh weight of the placental organs during the observed period in normal and anaemic pregnancies.
– · – · – · – = Yolk sac placenta of normal pregnancy.
——— = Allantoic placenta of normal pregnancy.
. . . . . = Yolk sac placenta of anaemic pregnancy.
· · · = Allantoic placenta of anaemic pregnancy.

that the values for the normal group rise above those of the anaemic group. This is also the case on the 20th and 21st days of pregnancy. The values for the anaemic pregnancy group are significantly lower than the corresponding values for the normal group on the 19th, 20th and 21st days of gestation (see Table XII and Fig. 12).

The content of Hy Fe per unit weight of the *all. placenta* at the different stages of gestation examined do not show any distinct increase with gestational age, either, although the values for the 20th and 21st days are somewhat lower than that of the 17th. The difference between values for the 17th day (1.78) and the 21st day (1.40) is not statistically significant ($P > 0.05$). (Table XII and Fig. 13.)

The value for the anaemic pregnancy group on the 15th day of gestation is significantly higher than the corresponding value for the *normal group* (Table XII). On the 17th day the concentration values of the two main groups are still approximately the same, but from then on the normal values seem to be higher than the anaemic ones, the differences being statistically significant on the 19th day of gestation ($P < 0.001$) and probable on the 21st day ($P < 0.01$).

It seems very likely that a larger experimental material would show a significant difference on the 20th and 21st days as well.

The *Y.S. placenta's* content of Hy Fe per unit weight at the different stages of pregnancy examined do not show significant differences. The differences noted could be caused by random deviations from the same value. Variance analysis gives $P > 0.05$ (Table XII and Fig. 13). Comparing these values with those of the *normal pregnancy group*, the values are found to be everywhere lower, the differences being statistically significant (Table XII).

Thus the two placental organs, whether considered together as the total placental barrier or individually, do not exhibit any significant variation during the whole of the gestation period examined, only a very slight tendency towards a decrease in Hy Fe concentration on the last days of pregnancy.

It should be noticed that the all. placenta of the anaemic pregnancy group has a higher content of Hy Fe per unit weight on the 15th day of gestation than that of the normal group, while the concentration is approximately the same on the 17th day, and lower on subsequent days observed. This is not the case with the Y.S. placenta, where the concentration is higher in the normal pregnancies at all observed stages of gestation (Table XII). Consequently, the Hy Fe per unit weight of the total placental barrier on the 15th day of gestation in the anaemic pregnancies is equal to that of the normal pregnancies, but a significant difference appears on the last three days of gestation. The physiological decrease in the normal values on the 20th and 21st days does not reduce them to the low level of the anaemic ones.

It may be seen from the absolute amounts of Hy Fe given for the placental organs (see Table IX) and the concentration values for the last two days of gestation, that no appreciable increase in weight occurs such as that observed in the normal pregnancies during the same period of gestation.

D. The relationships between the concentration of Hy Fe in the foetus and its placental structures under the given experimental conditions

There are two possible ways of considering the concentration relations between the foetus and the placental tissues: The relations may either be expressed in terms of the total concentration of foetal

iron or in terms of the concentration of Hy Fe. However, the total concentration of foetal iron will hardly stand in any intimate relation to the concentration of placental Hy Fe, since it includes iron compounds presumably of less importance in these respects. The transfer of iron from mother to foetus involves Hy Fe (maternal serum iron — placental transfer iron) and the corresponding iron fraction of the foetal organism may be expected to react more sensitively to variations in the transfer mechanism. It is true that the greater part of the foetal Hy Fe is the storage-iron of the foetal body, but this fraction is supposed to reflect the iron balance of the foetus in that the transportation of sufficient iron to the foetus is indicated by well filled iron depots, and an insufficient transfer by depletion of the stores of foetal body iron. In accordance with these considerations the concentrations of Hy Fe in the foetal tissues are compared with the corresponding concentrations of the placental organs.

1. The normal pregnancy group

a. *Foetus — total placental barrier*

In the normal pregnancy group the concentration of Hy Fe in the *total placental barrier* is higher than that of the foetal tissues during the period from the 15th to the 19th day of pregnancy. The differences are statistically significant or probable. On the 20th and 21st days of gestation, however, the reverse situation is noted. The amount of Hy Fe per unit weight of the total placental barrier then falls below the corresponding value for the foetal body, the difference again being statistically significant (see Tables XI and XIII).

b. *Foetus — all. placenta*

The concentration of Hy Fe in the all. placenta, like that for the total placental barrier, is higher than the concentration in the foetus during the whole period from the 15th to the 19th days of gestation, though not in the same degree, the difference not being statistically significant (see Table XIII). The foetal values increase on the 20th day of gestation and remain unchanged on the 21st day, whereas the reverse is true for the concentration in the all. placenta. The differences which arise are statistically significant, the foetal values being higher than those of the all. placenta (Table XIII).

Table XIII.

Mean individual differences between the concentrations of Hy Fe in the foetus and the placental structures, and between the two placental organs in normal and anaemic animals. (The values on which the differences are based are given in Table XI.)

Day of pregnancy	No. of diff.	Mean individual difference between			
		Foetus and total placental barrier	Foetus and all. placenta	Foetus and Y.S. placenta	Placenta and Y.S. placenta
		Normal pregnancy group			
15	15	-0.41 ± 0.10**	-0.16 ± 0.06*	-1.16 ± 0.19**	-1.01 ± 0.20**
17	18	-0.40 ± 0.08**	0.13 ± 0.08	1.43 ± 0.22**	1.30 ± 0.22**
19	17	-0.38 ± 0.13*	-0.11 ± 0.18	-1.08 ± 0.14**	-0.97 ± 0.24**
20	15	$+0.51 \pm 0.06$**	$+0.73 \pm 0.10$**	-0.03 ± 0.20	-0.72 ± 0.20**
21	18	$+0.39 \pm 0.12$*	$+0.59 \pm 0.14$**	$+0.01 \pm 0.16$	-0.58 ± 0.17**
		Anaemic pregnancy group			
15	10	-0.74 ± 0.07**	-0.75 ± 0.07**	-0.68 ± 0.10**	$+0.07 \pm 0.11$
17	9	-0.72 ± 0.13**	-0.63 ± 0.11**	-0.83 ± 0.18**	-0.20 ± 0.18
19	10	-0.28 ± 0.07*	-0.32 ± 0.09*	-0.19 ± 0.18	$+0.13 \pm 0.22$
20	10	-0.09 ± 0.20	-0.04 ± 0.25	-0.27 ± 0.23	-0.23 ± 0.33
21	10	-0.35 ± 0.11*	-0.26 ± 0.13	-0.52 ± 0.14*	-0.26 ± 0.18

N. B. ** the difference is statistically significant.
* the difference is statistically probable.

c. *Foetus — Y.S. placenta*

The same features are encountered in the relationship between the foetal Hy Fe concentration and that of the Y.S. placenta, i.e. the Y.S. placenta values are higher by a statistically significant amount than those of the foetus in the period from the 17th to the 19th days of gestation (Table XIII). The Y.S. placenta values decrease by a relatively large amount, whereas, as previously mentioned, there is an increase in the foetal values during the following two days of gestation. The difference observed at the earlier stages of gestation thus disappears completely on the 20th and 21st days of gestation. Foetus and Y.S. placenta then have approximately the same concentration values (Table XI).

d. *All. placenta — Y.S. placenta*

It is obvious from Table XI that the concentration of Hy Fe in the Y.S. placenta is higher than that of the all. placenta, throughout

the material examined and in all observed stages of gestation. The variation of the concentration is however the same in both structures — a gradual increase from the 15th to the 19th day of gestation, followed by a decrease. (Table XIII and Fig. 13.)

e. *Summary*

Regarding the relationship between the concentration of Hy Fe in the foetus and in the placental tissues at the different stages of gestation examined, it may be stated that the concentrations in the placental structures are higher than that in the foetus from the 15th to the 19th days of pregnancy. But from then there is a decrease in the placental values, accompanied by an increase in the corresponding foetal concentrations, leading to a gradient in the opposite direction, the concentration of Hy Fe in the foetus becoming higher than that in the placental organs during the 20th and 21st days of pregnancy. These features are more or less the same for each of the two placental organs referred to. However, it should be noted that the concentration of Hy Fe in the *Y.S. placenta* is so high that the increase in the corresponding foetal concentration on the 20th and 21st days of gestation does not suffice to raise the foetal values above those of the *Y.S. placenta*, though these latter are decreasing towards term.

As regards the *all.* and *Y.S. placentas*, the latter maintains throughout a higher concentration of Hy Fe, though both placental organs show increasing concentration up to the 19th pregnancy day, followed by a decrease on the two remaining days.

2. The anaemic pregnancy group

a. *Foetus — total placental barrier*

The concentration of Hy Fe in the *total placental barrier* is higher throughout the observed period of gestation than that of the foetal tissue (Table XIII). Even in this group there is an increase of the foetal concentration of Hy Fe up to the 20th day, but this is not sufficient to bring the concentration to the level for the total placental barrier. The differences are most pronounced between the 15th and 17th days of gestation — when they are statistically significant — but less so on subsequent days. Table XIII and Fig. 10 C.

The variations are thus much the same in the normal and anaemic pregnancy groups, the only difference being that in the anaemic group the foetal concentration does not reach or rise above that of the total placental barrier during the last two days as it does in the normal pregnancies. The difference between the anaemic and normal groups in this respect is significant ($P < 0.003$).

b. *Foetus — all. placenta*

The same applies to the relationship between the concentration of Hy Fe in the foetus and in the all. placenta, i.e. the foetal values are everywhere lower than those for the all. placenta, the difference being greatest between the 15th and 17th days of pregnancy, when it is statistically significant. From then on the difference diminishes during the 19th and 20th days, not being statistically significant on these days (Table XIII). The main difference between the normal and anaemic groups in this respect arises from the above-mentioned less pronounced increase, or even absence of any increase, in the concentration of Hy Fe in the foetus during the last two days of gestation in the anaemic pregnancy group. For this reason, the foetal concentration does not rise as high as that in the all. placenta. This difference between the two main groups is statistically significant on the 20th and 21st days of gestation ($P < 0.003$).

c. *Foetus — Y.S. placenta*

A comparison between the concentration of Hy Fe in the foetal tissues and the Y.S. placenta results in approximately the same observations as those made for the normal pregnancy group. From the 15th day of gestation to the 17th the foetal values are significantly lower than those of the Y.S. placenta (Table XIII). At the subsequent stages observed the foetal values are still lower than the corresponding Y.S. values, though no longer in a statistically significant manner. There is no appreciable difference between the normal and anaemic groups in this respect. The difference between values for the foetus and for the Y.S. placenta is somewhat greater in the normal pregnancy group between the 15th and 19th days of gestation, and in the anaemic group on the 20th and 21st days.

d. *All. placenta — Y.S. placenta*

As previously mentioned, the concentration of Hy Fe in both the all. placenta and the Y.S. placenta do not show any appreciable trend. Furthermore, the values are approximately equal (Table XI and Fig. 13). No statistically significant differences could be found between them (Table XIII). The differences with respect to the corresponding normal observations will be obvious from this.

e. *Summary*

Regarding the above observations on the relations between the concentrations of Hy Fe in the foetal and placental tissues of the anaemic pregnancy group, and comparing them with the corresponding features in the normal pregnancy group, we have the following.

In the *anaemic group,* the concentration of Hy Fe in the total placental barrier is higher than that in the foetal tissues throughout the observed period of gestation, the difference being most pronounced between the 15th and 17th days period, when it is statistically significant. The difference becomes smaller during the last three days of pregnancy. The same result is obtained for the two separate placental organs in comparison with the foetal tissues.

The concentration in the *all.* and *Y.S. placentas* are approximately the same at all the observed stages of gestation.

Comparing these findings with those for the *normal group*, it should be noted that the concentration of Hy Fe in the foetus, which does not increase in the same way as the corresponding normal value on the final days of pregnancy, does not rise above the individual placental concentrations or that of the total placental barrier.

On the final days of normal pregnancy the *Y.S. placenta* has about the same concentration of Hy Fe as the foetus. In the anaemic group, however, the Y.S. placenta shows a tendency to higher concentration than the foetus during the observed gestation period.

In the normal group the *Y.S. placenta* has a higher concentration of Hy Fe than the *all. placenta* at all observed stages of gestation. Both concentrations exhibit a definite increase up to the 19th day of gestation and then decrease towards term. In the anaemic group, on the other hand, the Hy Fe per unit weight of the two placental organs remains approximately stationary at a level far below the normal values.

CHAPTER IX

Discussion of the experimental results and general discussion of the transfer of iron from mother to foetus

The mean amounts of total and Hy Fe in the rat foetus on the 21st day of normal pregnancy are 0.21 and 0.09 mg respectively, according to the present measurements (see Table IX). It should be noted, however, that these figures apply to the rat foetus at the beginning of the last day of pregnancy. There are then another 12 hours or so before parturition, during which time more iron would presumably accumulate in the foetal organism, to judge from the pronounced increase in the iron content of the foetal body observed during the last days of pregnancy.

With these reservations, the values obtained are in good agreement with those given by other authors. ALT, 1938, found the total iron content of the newborn rat to be about 0.25 mg, and LINTZEL, RECHENBERGER, and SCHAIRER, 1944, arrived at approximately the same value. Somewhat higher figures were given be WIDDOWSON and MCCANCE, 1948, 1951, (about 0.35 mg), while HUGGET and WIDDAS, 1951, gave a value closer to those of ALT and LINZEL and coll., — to judge by their diagrams, the total iron content of the newborn rat lies within the range 0.25—0.30 mg.

As regards the 'non-haemoglobin' iron of the foetal body, ALT, 1938, obtained an approximative value of about 0.12 mg by subtracting the calculated amount of iron in the estimated foetal haemoglobin from the total iron of the foetus. LINTZEL and coll., 1944, arrived at a somewhat higher value by determining the foetal haemoglobin iron acc. to the method described by LINTZEL, 1930, 1933. With the reservations concerning additional iron accumulation in the foetus during the last 12 hours or so before parturition, these values seem to correspond rather well with that of 0.09 mg obtained in the present investigation for the 21st day of normal pregnancy. They all constitute approximately half of the total iron values reported by the same authors.

The present results also show that this relationship between the amount of total and Hy Fe in the foetus is practically constant throughout the period of pregnancy examined.

The literature contains no other reports of determinations of these foetal iron fractions at different stages of gestation as far as I know.

It is clear from the results of the present investigation that both the total Fe and the Hy Fe — the two fractions related to one another in the above unchanging ratio, — increase rapidly as gestation advances. Some two-thirds of the foetal iron found on the 21st day of normal pregnancy was delivered in the course of the 19th and 20th days. As already mentioned, it has been pointed out by VAHLQUIST, 1944, and RATH and coll. 1950, that in human pregnancy the main part of the foetal iron is provided during the last three months. The same appears to apply to the rat, if we take into account the very short gestation period, — the last three days or so of the rat's pregnancy apparently correspond to the third trimester of human pregnancy in this respect. According to IOB and SWANSON, 1938, the amount of total iron per unit foetal weight does not vary during the period of development in human pregnancy, if calculated for tissue free of fat and water. Calculating upon a basis of fat-free substance, WIDDOWSON and SPRAY, 1951, found an increase with development, however. HUGGET and WIDDAS, 1951, examined a single rat foetus on the 14th day of gestation, and obtained a value (about 8 mg iron per 100 gm wet foetal weight) considerably higher than that obtained by them immediately before and after parturition (5—6 mg of iron per 100 gm wet foetal weight). They therefore suggested that the amount of total iron per unit foetal weight decreases as gestation progresses. The corresponding mean value obtained by the present author for the 21st day of normal pregnancy is 5.6 mg of total iron per 100 gm of fresh foetal weight. This is in good agreement with the values obtained by LINTZEL and coll., 1944, and MCCANCE and WIDDOWSON, 1951 (5.18 and 5.76 gm respectively). The latter authors further state that the total iron content per 100 gm of fat-free weight does not lie outside the range 5.70—6.97 for the newborn of any other species than the rabbit and the pig, and that this range also holds for adults.

The present results show that the amount of total iron per unit foetal weight increases from the 15th day of gestation until the 20th day. The value given by HUGGET and WIDDAS for the 14th day is nearly 4 times as high as that obtained by myself for the 15th day of normal pregnancy, and must be considered incorrect.

From the 20th day on, the further accumulation of iron in the foetal body, though occurring at the highest rate noted, was accompanied by an increase in foetal weight large enough to keep the total iron per unit foetal weight at the same level. Judging from the values

of total iron per unit foetal weight given by the authors cited above, the same applies to the period of gestation immediately before parturition, which was not observed in this investigation. Recalling the fact that the Hy Fe of the foetus constituted approximately half the total iron content of the foetus throughout the period of normal pregnancy observed, it follows that the Hy Fe increases in the same way with advancing gestation, both in the absolute sense and per unit foetal weight.

It seems reasonable to conclude that as gestation progresses the rat foetus accumulates iron, both in the absolute sense and per unit weight, at an increasing rate. From the 20th day on, the very considerable increase in the absolute amount of iron is balanced by an increase in foetal weight, so that the iron content per unit foetal weight remains unchanged.

The iron accumulated by the foetus was shown to serve chromoprotein synthesis and depot formation in about the same degree, assuming the Hy Fe *measured in the present investigation to represent most of the storage-iron fraction.* This is in conformity with the findings of Lintzel and coll., who considered the amount of total iron in the newborn rat to be about 0.25 mg and the haemoglobin iron of the foetus to be 0.10 mg, which means that more than 50 % of the total iron of the foetal tissues is in a 'non-haemoglobin' form.

As far as I know, no determinations of iron in the placental organs have been carried out, except for those briefly reported by Vosburg and Flexner, 1950. These authors determined the concentration of iron in the placenta of the guinea pig, washed free from blood, and found it to average 57 μg per gm of tissue. It was not reported whether the *all. placenta* alone or both this and the *Y.S.* were included in the analysis. In the present investigation no value of this magnitude was obtained for either of the placental organs of the rat. As earlier pointed out, the present author seriously doubts the possibility of washing the placental organs free from blood. Not even viviperfusion accomplishes this.

The results reported for normal pregnancy in the present investigation have shown that considerable amounts of iron occur in both the placental structures, the *all.* as well as the *Y.S. placenta.* The author's method was shown not to liberate blood iron in significant amounts under the given experimental conditions. *The measured iron must therefore be attributed entirely to the placental tissues, and the experimental results consequently reveal the presence in the placental*

organs of some hydrolysable, acid-soluble iron compound, or compounds, demonstrable throughout the observed period of gestation.

The amounts of Hy Fe in the placental organs were shown to *increase with advancing pregnancy*. This might very well be expected, as the rate of transfer of iron to the foetus increases with foetal development. *But in the very period when the main part of the foetal iron is delivered no further accumulation of Hy Fe occurs in the placental tissues*. The above-mentioned increase in placental iron lasts only up to the 19th day of pregnancy, and from then on the placental content of Hy Fe remains at the same level. It was further found that the main part of the iron in the placental tissues occurs in the larger placental organ, the *all. placenta*, the *Y.S. placenta* containing only half as much throughout the experimental period. It is interesting to note that the accumulation of Hy Fe follows the same course in both the placental organs, reaching a maximum on the 19th day of pregnancy. This variation is more marked when the concentration of Hy Fe per unit fresh weight of the tissues is considered. The concentration of Hy Fe thus obtained increases continuously for both the placental organs, up to the 19th day of pregnancy. From then on, however, *a decrease is observed for the remainder of the experimental period*.

It is further obvious from the concentration values that throughout the experimental period, the *Y.S. placenta* contains considerably more iron per unit weight than does the allantoic structure. But as with the absolute amounts, the changes in Hy Fe concentration with advancing gestation in the two placental organs are parallel.

Comparing the corresponding concentration values of the foetus with these for the placenta, we get a better view of the changes in the placental iron with respect to the demonstrated changes in the foetal content of Hy Fe. In making this comparison, I have chosen to calculate the Hy Fe concentration of *both the placental organs taken together, considered as the total placental barrier*. It has been stressed earlier that the concentration values of the two placental structures, though different in magnitude, increase and decrease proportionately during the experimental period. The comparison shows that the concentration of Hy Fe of the total placental barrier is higher than that of the foetus up to the 19th day of pregnancy. But on the following two days the reverse is the case. We thus see that *during the period of greatest iron accumulation in the foetus, the amount of Hy Fe per unit*

fresh weight of the placental organs decreases significantly, and from being higher than those of the foetal body, it now falls below. If the two placental organs are considered separately, this is not obvious, because the concentration values of the *all. placenta*, though increasing, do not rise above those of the foetus in the earlier part of the experimental period, and the *Y.S.* values, though decreasing, do not fall below the foetal ones in the latter part.

The results obtained for the *anaemic group* are of interest in assessing the significance of the iron compound or compounds found in placental tissues at different stages of gestation. The haemorrhagic anaemia arrived at with the technique used was shown to be pronounced and constant throughout the experimental period. The maternal iron depots were almost depleted and the iron transferred to the foetus must be assumed to be supplied mainly from increased iron absorption in the maternal gut. It is shown that the iron content of the foetus is considerably less than that of the normal cases. An increase in foetal iron with advancing gestation was noted, though much less pronounced than in normal pregnancy. This was most clearly demonstrable between the last two observed days of gestation, *when the foetus received no appreciable amount of further iron.* The total iron content of the anaemic foetus on the 21st day was less than half the normal value. As was to be expected, the Hy Fe fraction was most reduced, to a value only one-third of the total iron.

The foetus of the anaemic mother was further shown not to increase in weight to the same degree as did that of the normal mother. It is not possible to say whether this was due to the deficient iron supply to the foetus or to some other deficiency brought about by the anaemic state of the mother.

Despite this curtailment of the increase in foetal weight, the amount of total Fe per unit foetal weight was lower than in the normal pregnancies, and this inferiority became more pronounced as gestation progressed. Though less pronounced than in normal pregnancy, there was an increase in the total Fe concentration with advancing gestation, the increase lasting until the 20th day of pregnancy. The concentration of Hy Fe in the foetus showed the same tendency until this stage of gestation, but contrary to the case with normal pregnancy, *the concentration decreased between the last two days observed.* This was due to the cessation at this stage of iron transfer to the foetus. *The foetus of the anaemic mother is in a state of "iron-*

hunger" which increases with advancing gestation and becomes most pronounced at the end of pregnancy, when the need for additional iron is most pronounced. At this final stage of pregnancy the rat mother is incapable of delivering more iron to the foetus.

The presence of Hy Fe in the placental tissues is still demonstrable under these conditions. At the earliest stage of the experimental period, when the foetal iron needs are rather insignificant, the content of Hy Fe in the placental organs is much the same as in normal pregnancy. But from the 17th day on the values start to fall below the normal ones. The content of Hy Fe describes proportionally, though in a much less pronounced way, the same increase in amount with gestational duration including the 19th day as was found in normal pregnancy. During the last three days of the experimental period the amount of Hy Fe in the placental tissues remains at approximately the same level, but far below the normal one.

Another respect in which anaemic pregnancy differed from normal pregnancy was the amount of Hy Fe found in the *Y.S. placenta.* This was shown to be a far smaller part of the placental iron in the earlier stages of the experimental period, but gradually accumulated more Hy Fe until it contained about one-third of the Hy Fe in both the placental organs on the 21st day of pregnancy. As mentioned earlier, this was the case throughout the experimental period in normal pregnancy.

If the amount of *Hy Fe per unit weight* of placental tissue is considered, it is found that the definite variation of concentration noted in normal pregnancy does not occur in the anaemic pregnancies. The increase in iron concentration up to the 19th day of pregnancy does not recur in the anaemic cases, and the decrease towards term observed in normal pregnancy is but faintly suggested in the anaemic pregnancy group. In the later part of the experimental period, the concentrations of Hy Fe in the placental organs are below those of normal gestation. This is the case for both the placental structures, which gave approximately the same concentration values throughout the experimental period.

Comparing the concentration of Hy Fe in the foetus with that in the total placental barrier, as was done for the normal group, it is seen that early in the experimental period the placental values were higher than the foetal ones, as was the case in normal pregnancy. Later, however, the values of the total placental barrier did not fall

below those of the foetus. This is mainly accounted for by the fact that the foetal values decrease towards the end of pregnancy, contrary to what happens in normal gestation.

Thus in the state of haemorrhagic anaemia arrived at, the foetal organism is inadequately supplied with iron, and the foetal iron deficiency becomes more pronounced as gestation progresses. The Hy Fe of the placental organs, expressed either absolutely or per unit weight of placental tissue, fall below the values noted for normal pregnancy as gestation progresses, and during the last part of the experimental period, when the iron deficiency of the foetus becomes most pronounced, this feature is most obvious. The characteristic changes noted for the iron contents of the placental organ in normal pregnancy were only faintly evident towards the end of the anaemic pregnancy. This was approximately true for both placental organs.

As already pointed out, the mechanism of the placental transfer of iron from mother to foetus is still unknown. The metabolic conditions with regard to iron differ on the two sides of the placental barrier. The maternal organism has adjusted its iron metabolism *to meet the requirements of the growing foetus by absorbing exogenous iron from the intestine in increasing amounts and by mobilising endogenous iron from the depots.* To this end the serum iron level is decreased and the iron-free transferrin concentration raised. The foetal organism, on the other hand, has an iron metabolism adapted to work *in the reverse direction.* Its iron depots are being built up at an increasing rate, and this feature of the metabolism manifests itself in a high serum iron level towards term — about twice as high as that of the mother — and in a transferrin concentration correspondingly low — half that of the mother. Towards the end of pregnancy, the placental transfer of iron from mother to foetus thus goes against a concentration gradient. Furthermore, the protein vehicle is supposed not to pass the placental barrier, judging by its concentrations in the maternal and foetal sera.

The hydrolysable tissue iron found in the placental organs must therefore represent some iron compound or compounds involved in the placental transfer of iron. This is indicated by its chemical properties, in that it is hydrolysable by the method used here and further by the definite relationship noted between its quantitative occurrence at different stages of gestation and the actual iron requirements of the foetus. *This means that the transfer process includes an accumulation*

of some hydrolysable iron compound or compounds in the placental tissues. The behaviour of the two separate placental organs appears to be similar in this respect, suggesting the same transfer principle for them both. Throughout the experimental period, the larger placental organ, the *all. placenta*, contains twice as much Hy Fe as the Y.S. one, but the amount of this iron per unit is appreciably less. However, both organs exhibit the same features of changes in their absolute and relative amounts of iron as gestation progresses.

As regards the nature of this iron fraction involved in the placental transfer, its hydrolysability and acid solubility suggest that all or part of it is contained in ferritin. This is supported by the findings reported in the histochemical part of this study, where ferritin was demonstrated to be present in both the placental organs.

The intestinal absorption of iron has been discussed in the review of the literature, and reference was there made to the existence of a regulated iron absorption, accomplished by what HAHN and coll. called 'the mucosal bloc.' Ferritin is known to be present in the intestinal mucosa, and has been associated with the intestinal uptake of iron. Whether it really takes an active part in the absorption or only expresses saturation of the intestinal mucosa with some other iron compound engaged in this respect is still obscure (GRANICK, 1951).

VOSBURGH and FLEXNER, 1950, suggested that the transport of iron across the placenta could be regarded as "a rather complex regulatory mechanism which may be analogous to that concerned with the absorption of iron from the gastro-intestinal tract." They also point out that the presence of ferritin in the placenta was demonstrated by LATHAM and VOSBURGH, though their findings are not yet published.

The results obtained in the present investigation confirm the presence of ferritin in the *all. placenta*, but also show that it occurs in the *Y.S. placenta* as well. *The fact that ferritin was observed in the visceral yolk sac membrane is quite reasonable, as the yolk sac is part of the foetal fore-gut, and its epithelium therefore of an intestinal nature.* It seems very likely that the absorption of iron carried out by this membrane is identical with that of the ordinary intestinal mucosa. It has already been mentioned that the structure separating the maternal blood from the yolk sac cavity — Reichert's membrane — is considered to be a permeable structure built up of collagenous and polysaccharide material. Maternal serum might very well pene-

trate such a membrane and become available to the absorptive action of the visceral yolk sac epithelium.

As regards the *all. placenta*, there is no epithelium with such absorptive characteristics in the route of transfer across the placenta. The foetal chorionic epithelium is directly bathed by maternal blood. It has been stressed, however, that the essential features revealed by the present investigation to be common to both placental organs suggest the same principle of iron transfer in both cases.

Measurements of the accumulation in the placental tissues of some hydrolysable iron compound or compounds — partly or wholely ferritin, — indicated some definite features. *The amount of this iron per unit placental weight in normal pregnancy increased with increasing foetal requirements of iron, but in the period of greatest foetal iron accumulation, — during the last two days of pregnancy observed, — it decreased.*

With severe iron deficiency in the maternal animal, rendering it incapable of fulfilling the iron demands of the foetus, the amount of placental tissue iron per unit weight remained approximately stationary and during the last two days of gestation definitely lower than in the normal pregnancies. This might be taken to indicate a *regulatory mechanism*. For the abundant maternal supply of iron to the foetal tissue reaches a maximum on the 19th day, as compared with the foetal need for the element. Under these conditions the placental tissues might become saturated with iron, as was the case with the intestinal mucosa. During the last two days of pregnancy, when the foetus requires most iron, the maternal iron supply might be more completely utilised, and the placental tissues consequently become more depleted of the intermediary iron compound involved in the regulatory process.

CHAPTER X

Experiments with radioactive iron

To check the results obtained from the chemical determinations of the amounts of Hy Fe in the placental structures and foetal bodies, some experiments with radioactive isotopes were carried out. It was

also considered of interest to investigate whether experiments of this kind could throw any additional light on the problems dealt with. It should be pointed out, however, that these experiments were no more than introductory, and were not considered to be conclusive, as some rather rough approximations had to be applied.

A. The test animals

These were divided up like the material already reported into two main groups, a *normal pregnancy group* and an *anaemic pregnancy group*. The two main groups were not subdivided according to different stages of pregnancy, however, but included rats from the last three days of gestation. The age and weight distribution of this material corresponded with that given for the material used in the chemical part of the investigation, as did the diet, the bleeding technique for the experimental anaemia group, and treatment in other respects. The total material consisted of 40 white, unmated female rats, equally distributed in the two groups. The material reported on consisted of 33 animals, of which 15 belonged to the *normal pregnancy group* and 18 to the *anaemic pregnancy group*.

B. Methods and experimental technique

The radioactive iron was obtained from Harwell, and contained the two radioactive isotopes of iron Fe^{55} and Fe^{59}, the latter predominating.

The radioactive material was received as a solution of ferric chloride of volume of 7.65 ml and with an iron content of 9.5 mg per ml. The total activity given on the day of delivery was 1 mC. The specific activity, 13.9 μC per mg of iron, was therefore fairly low. The iron was transformed into ferrous ascorbate and the injection solution was prepared as required during the experiments by dissolving the ascorbate in 5 % glyco-physiological saline to obtain an iron concentration of 1.44 mg per ml and an activity of 20 μC per ml. The ferrous ascorbate was kept in vacuum and darkness.

Two injections were given, one rather large, 1.44 mg of iron, and one half as big, 0.72 mg, with activities of 20 and 10 μC respectively. The injections were given in the morning, half the dose was injected into the exposed femoral vein of the pregnant rat and after 2 hours the other half of the dose was injected into the exposed femoral vein of the other leg. The animal was killed after another 2 hours. The injections were performed under light ether anaesthesia. The animals were again anaesthetised with ether 4 hours after the first injection of the radioactive salt. A blood specimen was obtained by heart puncture, and

the foetal bodies and placental tissues were dissected out in accordance with the procedure already described. Strict precautions were taken against contamination of the foetal structures with maternal blood. The different tissues were carefully washed in cold physiological saline until this rinsing solution became colourless. One uterine swelling representing the upper egg of the right uterine branch was not included in this treatment but was cut out as a whole and put in a vessel with chilled methanol. These specimens were used for autoradiographic studies, of which a short account has been given. The blood specimen was centrifuged at 3 000 r.p.m. for 10 min. and a measured volume of plasma pipetted off into a Kjeldahl vessel. The well rinsed foetal and placental structures were weighed in close weighing vessels. The yolk-sac and placental tissues were transferred directly to Kjeldahl vessels, but the foetal bodies were homogenised in an amount of bidistilled water equal to their weights and 15 ml of the homogeneous suspension transferred to a Kjeldahl vessel. The biological material thus obtained was ashed according to the methods of wet ashing already described. When ashing of the organic matter was complete, the clear contents of the Kjeldahl vessel were transferred to a volumetric flask and made up to 25 ml with bidistilled water. The subsequent procedure was in strict accordance with the methods described by Vosburgh, Flexner, and Cowie, 1948, and modified by Naeslund, 1951, the principle of which is that the iron is taken up in ammonium oxalate solution and deposited electrolytically from the ferric ammonium solution obtained on to a copper disc cathode. This disc is then brought close to the mica window of the Geiger-Müller tube connected with the impulse counter. (To 2 ml of the diluted solution obtained from ashing were added 2.58 mg carrier iron. The iron of the solution was precipitated by adding 40 % sodium hydroxide until the solution became neutral, using phenol red as indicator. The precipitate was separated with the centrifuge and dissolved in 6 N hydrochloric acid, the solution being neutralised with the requisite amount of 25 % ammonium hydroxide. Some 10 ml of saturated ammonium oxalate solution and a few drops of saturated oxalic acid were added, and the solution transferred to the electrolytic cell, complete transfer being ensured by repeated rinsing of the previous vessel. The electrolytic apparatus was built after Naeslund's description, and the electrolysis performed in accordance with his recommendations. Tests for the presence of free iron remaining in the solution were made by means of the o-phenantroline reaction.)

When the electrolysis was complete and the copper disc dried in a thermostat chamber, the disc plated with iron was rigidly fixed upon a slide and brought into a central position immediately beneath the window of the counter tube. The Geiger-Müller tube used was of the type LKB 5006, a self-quenching, bell-shaped beta-tube, filled with argon and alcohol vapour. Its mica window, 30 mm in diameter, weighed 3.4 mg per sq. cm. This tube is insensitive to the radiation produced by the disintegration of Fe^{55} and records only the radiation of Fe^{59}.

The electronic scaler was manufactured by LKB.

The counting rates for the different specimens were determined and the background rates subtracted. The total number of disintegrations counted varied with the activity of the sample, but was never below 5 000 c.

C. Discussion of methods and experimental technique

1. *The injection doses.* It will be noted that rather heavy doses of iron were given to the rats, far larger than the tracer doses used in research work upon placental permeability. However, the purpose of this complementary work was mainly to establish whether or not there is some sort of regulation mechanism for the iron transfer to the foetus, and whether such a regulation, if it exists, undergoes a change in experimental anaemia. The doses of iron administered were suitable for this purpose.

The radioactive iron was administered in a manner that would bring about a rather high concentration of radioiron in the maternal serum during the period of the experiment, the purpose of this high concentration being to exclude the possibility of iron returning from the foetus to the maternal circulation affecting the results obtained. The activity of the plasma in the animals injected with the larger dose (1.44 mg Fe), corresponded to a mean value of 2 250 γ % of iron far above the capacity of the serum to bind it and consequently for the greater part in the form of 'loosely bound' iron in the serum. The corresponding value for the animals injected with the smaller dose (0.72 mg of iron) was 800 γ %, so that a greater part of the injected iron was incorporated in the transferrin-iron complex (the total iron-binding capacity of the serum being about 500 γ % in the rat, acc. to KINNEY, HEGSTED, and FINCH, 1949). WALLENIUS studied these plasma samples by means of the paper strip electrophoresis method and examined the papers used radiographically. He showed that the radioactive iron occurred in the β_1 globulin fraction of the plasma, and this was the case in samples from rats with either injection dose.

The animals showed no toxic symptoms during the experiments. The injections were performed under light ether anaesthesia. This has been shown by FLEXNER & ROBERTS, 1939, not to affect the placental permeability.

2. *Amount of carrier iron co-plated in the electrolysis.* VOSBURGH and FLEXNER, 1950, using the same technique and the same type of Geiger-Müller tube, found no significant self-absorption of radioactivity with up to 5 mg of co-plated carrier iron. In view of this result, I chose to work with about 3 mg of carrier iron.

3. *The layer of iron deposited on the copper disc.* This was always uniform. Moreover, an autoradiograph of the iron on a representative disc showed the layer to be homogeneous. (See Fig. 14.)

Fig. 14. Representative autoradiograph of a copper disc electroplated with iron.

D. Expression of the results

The results are presented as counts min./gm of tissue, as a percentage of the injected dose. These values are referred to in the following as the "specific activities" of the structures in question. The values obtained for the foetal body, the yolk sac and the placenta, are summed up to a total. *The percentage of the activity which arises from a particular structure is referred to as the "relative activity" of the structure.*

The activity of the original injection was attained by measuring the activity of a 3 : 312 500 dilution of the original solution, three samples of this kind being used as standards and included in every measurement. These standards were also used to correct for the decay of radioactivity.

E. Calculation of the activity of the placenta freed from blood

The total amount of iron in the placenta on each of the three days of gestation was determined as the mean of three determinations in each experimental group. The values of haemoglobin iron could be calculated by subtract-

Table XIV.

Percentage weight of whole blood in the placentas on the last three days of normal and anaemic pregnancy.

Day of gestation	Normal pregnancy	Anaemic pregnancy
19	17.9	24.3
20	21.3	23.2
21	22.6	27.7

Table XV.

Percentage weight of plasma in the placentas on the last three days of normal and anaemic pregnancy.

Day of gestation	Normal pregnancy	Anaemic pregnancy
19	10.2×1.03[1]	17.9×1.03
20	12.4×1.03	16.4×1.03
21	13.2×1.03	19.6×1.03

[1] The specific gravity of plasma is assumed to be 1.03.

ing the previously reported values of Hy Fe in the corresponding placentas from the values of total Fe. Assuming the iron content of haemoglobin to be 0.335 per cent by weight, the amount of haemoglobin in gm may be computed. The values of the haemoglobin concentration of the blood obtained for the different days of gestation and the different experimental groups — dealt with in an earlier section — may be used for an approximate evaluation of the amount of blood in the different placentas expressed as a percentage weight of the organs, if the specific gravity of the blood is taken to be 1.06. Furthermore, the haematocrite values for the different days and groups of pregnancy available in the above material make it possible to calculate the amount of plasma in ml included in the placentas of the different rats, and to express it as a percentage of the placental weights. The activity of the placenta free from blood may thus be estimated by subtracting the number of counts per min. corresponding to the calculated amount of plasma included in the placenta from the number of counts per min. for the placental tissue as a whole. To calculate the activity per gm of tissue, the activity thus obtained is divided by the blood-free placental weight. The approximate values of the percentage weight of blood and plasma in the different placentas are given in Tables XIV and XV. The weight of plasma is given as the product of the volume of plasma and its assumed specific gravity, 1.03.

These calculations involve some rough approximations. To begin with, the haematological values obtained from the heart blood given in the previous part of this study are applied to the placental blood. This may involve an error, especially as part of the placental blood is of foetal origin. However, this method

of computation was preferred to that of endeavouring to free the placental tissue from blood by rinsing or viviperfusion of the organ. As pointed out previously, this latter method is not at all reliable, as a considerable part of the blood may be left in the placental tissue, while at the same time radioactive material in the tissue proper might be dissolved by this treatment. Some of the results indicate that the reduction of the placental activity brought about by subtracting a calculated amount of plasma activity from the total activity of placental tissue is not too small, as the 'relative activities' obtained are very low (see Table XVI N: 7, A: 5, A: 12, A: 14, and A: 19.)

F. Experimental results

The results expressed as 'relative activities' (defined above) are given in Table XVI. As the size of the injected dose was proved statistically not to affect the values given, the results of Table XVI are presented in only two groups, the *normal* and the *anaemic pregnancy groups*.

1. The normal pregnancy group

The mean distribution of the radioactive iron in the different parts of the conceptus was 24 % in the foetal body, and the remaining 76 % concentrated in the placental organs, i.e. the *Y.S.* and the all. placenta. In 4 of the 15 rats examined the foetal organism contained more than 25 %, and in 2 rats, more than 40 % of the total activity of the egg. The two principal placental tissues, the *Y.S.* and the all. placenta, taken together as the total placental barrier, thus accounted for some 3/4 of the activity, the Y.S. placenta contributing about 47 % and the all. placenta 29 %. (In only 3 of the animals examined did the all. placenta contain percentually more of the total activity than the *Y.S.*) Under the experimental conditions applying, the radioactive iron injected intravenously was thus after four hours concentrated to the placental organs to a degree three times as great as the concentration in the foetal organism, the greatest accumulation being in the Y.S. placenta.

2. The anaemic pregnancy group

The mean foetal part of the activity from the egg was 37.5, the remaining 62.5 % being contributed by the placental structures. Thus, compared with the normal pregnancy group, the foetal organism of the anaemic group contains a larger part of the activity unit, the difference being statistically probable ($P < 0.02$). There is a change

Table XVI.

The relative activities of the foetal bodies and placental structures during the last three days of normal and anaemic pregnancy.

XVI a.

Normal pregnancy				
No.	Foetus	Y.S. placenta	All. placenta	Total plac. barrier
1	14.9	49.5	35.6	85.1
2	19.9	49.9	30.2	80.1
3	15.9	46.4	37.7	84.1
6	18.6	24.7	56.7	81.4
7	21.8	69.8	8.4	78.2
8	22.9	44.9	32.2	77.1
9	23.0	31.9	45.1	77.0
10	30.3	52.1	17.6	69.7
11	18.4	58.8	22.8	81.6
12	44.1	29.1	26.8	55.9
13	24.8	43.8	31.4	75.2
15	7.6	70.8	21.6	92.4
16	34.0	41.0	25.0	66.0
17	13.3	75.6	11.1	86.7
18	51.6	21.8	26.6	48.4
Mean	24.1	47.3	28.6	75.9

XVI b.

Anaemic pregnancy				
No.	Foetus	Y.S. placenta	All. placenta	Total plac. barrier
2	29.3	10.2	60.5	70.7
3	25.5	31.5	43.0	74.5
4	53.8	32.8	13.4	46.2
5	58.5	35.8	5.7	41.5
6	16.7	41.6	41.7	83.3
7	16.6	28.1	55.3	83.4
8	59.3	28.8	11.9	40.7
9	21.1	25.7	53.2	78.9
10	39.5	6.8	53.6	60.4
11	37.9	44.7	17.4	62.1
12	17.8	76.6	5.6	82.2
13	36.6	29.0	34.4	63.4
14	60.0	32.0	8.0	40.0
15	35.9	21.0	43.1	64.1
16	13.5	30.0	56.5	86.5
17	46.9	39.8	13.3	53.1
18	47.1	16.1	36.8	52.9
19	59.5	35.6	4.9	40.5
Mean	37.5	31.5	31.0	62.5

in the distribution of 'relative activities' which favours the foetal organism at the expense of the total placental structure. Regarding the proportion of the 'relative activities' of the two principle placental organs, this is also altered, in the direction of an equalization between the Y.S. and the all. placenta.

The injected radioactive iron, as it manifests itself in the 'relative activities' of the different parts of the egg, thus accumulates in the placental organs to a degree only 1.7 times greater than the concentration of iron in the foetal body, the Y.S. and the all. placenta containing approximately the same concentration.

G. Discussion

The results of the investigation by overloading the organism with radioactively labelled iron on the last three days of normal and anaemic pregnancy bear out the results obtained from the chemical experiments. The high concentration of Hy Fe in the Y.S. — and to a lesser degree in the all. placenta — in normal pregnancy corresponds to an accumulation of labelled iron in these structures, which is most pronounced in the Y.S.. The iron received by the foetus during the four hours before its removal is only one-fourth of the iron contained in the egg, the rest being contained in the two placental organs. This supports the theory earlier arrived at that the passage of iron through the placenta involves an accumulation of iron (in some iron-protein complex) in the placental tissues, indicating a rather complex transfer mechanism which might well be of a regulative nature. It may be seen that this accumulation of labelled iron in the placental structures is less pronounced in experimental anaemic pregnancy, as the foetus, in a condition of 'iron-hunger,' receives more iron. During normal gestation, the Y.S. contains the greater part of the labelled iron in the total placental barrier, whereas in the corresponding situation of experimental anaemia this difference between the two placental organs disappears, the allantoic placenta containing a concentration of labelled iron equal to that of the Y.S. in this case. These observed features support the theory of a regulative mechanism of iron transfer, which allows a speedier transfer in conditions of foetal iron deficiency, though a concentration of iron in the placental barriers is still involved. This concentration is presumably of fundamental importance in the process of iron transfer.

Summary

1. A survey is made of the literature concerning the main features of iron metabolism in non-pregnant and pregnant conditions, with special reference to the problem of iron transfer from mother to foetus.

2. Some morphological and physiological features of special interest concerned with the placental organs of the rat are mentioned and the placental function of the yolk sac is documented by literary references.

3. The test animals are divided into two main series, one representing normal pregnancy and the other iron-deficient pregnancy. Each series is subdivided into sub-groups representing different stages of the second half of gestation. The experimental anaemia is produced by daily bleeding. It was shown that the degree of anaemia maintained throughout the whole experimental period had been achieved on its first day. The significance of the blood loss is discussed briefly.

4. Histochemical investigation of the placental organs revealed the existence of an intense iron-reaction in the *fresh* specimen of the *visceral yolk sac wall* at all the stages of *normal pregnancy* which were examined. Using the ordinary histochemical technique upon *fixed material*, the iron reaction of the same tissue under identical experimental conditions was far less pronounced. The *allantoic placenta* did not give a positive iron reaction by the ordinary histochemical technique. In the *anaemic pregnancies* the iron reaction of both the fresh specimens and of the histological sections was considerably less pronounced.

It was further shown that *ferritin* occurred in appreciable amounts in the *visceral yolk sac wall* and in the same region that revealed the strongly positive iron reaction when treated fresh. *Ferritin* was also present in the *allantoic placenta* to a somewhat lesser degree. In corresponding animals of *anaemic pregnancy* hardly any ferritin at all was demonstrated in either of the placental organs.

It is thought that the positive iron reaction obtained in the *fresh*

yolk sac material was due to *ferritin*, and that the positive iron reaction of the *histological sections* was caused partly or wholly by some other iron compound, possibly *haemosiderin*. It is suggested that the poor iron reaction and ferritin content of the placental structures of anaemic pregnancy indicates that the iron compounds thus demonstrated were involved in the placental transfer of iron and reflect the iron lack of the mother.

5. Reichert's membrane separating the yolk sac cavity from the maternal blood was shown to reveal a positive iron reaction in the histological sections in all stages of normal pregnancy. In the corresponding anaemic conditions, however, this reaction was only faint or absent. The significance of this is discussed, and the conclusion drawn that *this membrane contains some iron compound which may be involved in the transfer of iron across the yolk sac placenta.*

6. Histological examination showed that Reichert's membrane is made up by collagenous and some polysaccharide material, which constitutes a permeable structure.

7. To determine the iron contents of placental and foetal structures a method was devised for measuring a certain iron fraction, here called the "*hydrolysable iron*". The exact origin and significance of this fraction is not clear, but it has been determined in all analyses, and it was definitely not contaminated by significant amounts of haemoglobin iron. Further, the total iron content of the foetus was always determined.

8. It is shown that as gestation advances the rat foetus accumulates iron at an increasing rate both in the absolute sense and per unit fresh weight. *The main part of the foetal iron is acquired during the last two days of gestation.* From the 20th day on the very considerable increase in the absolute amount of foetal iron is balanced by an increase in foetal weight, so that the iron content per unit foetal weight remains stationary. The hydrolysable iron fraction constituted about half of the total iron throughout the observations, and, assuming this fraction to represent most of the storage-iron of the foetus, the iron accumulated in the foetal organism seems to serve chromoprotein synthesis and depot formation about equally.

It was found that considerable amounts of hydrolysable iron occurred in both of the placental organs throughout the experimental period. The measured iron must be attributed to the *placental tissues, indicating the existence of some hydrolysable, acid-soluble iron compound*

or compounds demonstrable throughout the observed period of gestation. Both of the placental organs, following the same parallel course in the accumulation of hydrolysable iron, increase their content of this iron fraction both absolutely and per unit weight up to the 19th day. Subsequently no further iron is accumulated and a decrease is noted per unit placental weight during the remaining days of the observed period of gestation. *Thus in the period of the greatest iron-transfer to the foetus no further iron is laid down in the placental organs, the hydrolysable iron content of which remains at the level noted on the 19th day and the amount of hydrolysable iron per unit weight of which falls below the 19th day value.*

The foetus of the *anaemic mother* was shown to be in *a state of iron-deficiency which increases with advancing pregnancy, and becomes most pronounced towards term, when the need for additional iron is most pronounced.* The rat foetus under these conditions contained considerably less iron than the normal one, this deficiency becoming most obvious during the last days of the observed period of gestation. No further iron was transferred to the foetus between the last two measurements. The hydrolysable iron fraction was the more diminished.

In the anaemic conditions produced, the placental organs were still found to contain hydrolysable iron, and, in the earlier stages of the observed gestation period, in much the same amounts as in normal pregnancy. From the 17th day onwards, the anaemic values start to fall greatly below the normal, expressed either absolutely or per unit weight of placental tissue. *During the last part of the experimental period, when the iron-hunger of the foetus becomes most pronounced, this feature is most obvious.* The total amount of hydrolysable iron in the placental organs increases with advancing gestation up to the 19th day, though much less than was the case in normal pregnancy, and subsequently remain on the same level. The amount of this iron fraction per unit weight of the placental organs, however, does not vary according to the duration of pregnancy as in the normal animals, but remains more or less unchanged throughout the observations.

9. The conclusion is drawn that the hydrolysable iron found in the placental organs must represent some iron compound or compounds involved in the placental transfer of iron, to judge from its chemical properties and amount at different stages of gestation. *The transfer*

process thus includes an accumulation of this iron compound or compounds in the placental tissues.

Regarding the chemical properties of this iron compound and the demonstration of ferritin in the placental tissues it is suggested that it is *ferritin.*

The measurements of the accumulation in the placental tissues of some hydrolysable iron compound or compounds, partly or wholly ferritin, indicate *the existence of a regulatory mechanism of the iron transfer across the placental structures.*

10. Experiments with the radioactive iron isotope Fe^{59} support the finding that the concentration of iron to the placental organs is an integral part of the placental transfer of iron, and that a regulatory mechanism of iron transfer across the placental barriers exists.

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